EUROPEAN MASTER IN QUALITY IN ANALYTICAL LABORATORIES

APPLICATION OF BISMUTH MODIFIED DISPOSABLE SCREEN PRINTED CARBON ELECTRODE FOR METAL- PLANT THIOLS COMPLEXATION STUDIES

Master thesis by **Belachew Tolla Feyssa**

Barcelona, February 2010

APPLICATION OF BISMUTH MODIFIED DISPOSABLE SCREEN PRINTED CARBON ELECTRODE FOR METALS- PLANT THIOLS COMPLEXATION STUDIES

A Thesis Submitted to University of Barcelona
Faculty of Chemistry
In Partial Fulfillment of the
Requirements for the Degree of
European Master in Quality in Analytical Laboratories

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FEBRUARY, 2010

This master thesis has been accomplishedin by Belachew Tolla Feyssa in the Department of Analytical Chemistry of the Faculty of Chemistry of the University of Barcelona (UB) under the direction of Dr. Jose Manuel Diaz-Cruz.

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Barcelona, 8th February 2010

AKNOLEDGEMENT

For all his sincere devotion to help me for the accomplishment of this work much appreciation is expressed for my supervisor Dr.Jose Manuel Diaz -Cruz.

I also gratefully acknowledge Prof. Miquel Esteban and Dr. Cristina Arino for their assistance and support during my study.

I would also like to express my gratitude for financial support provided by European Commission to complete this master's course.

Special thanks are due to Dr. Elena Checkmeneva for her unlimited support and encouragement. Also I can't forget the help of Dr.Olga Gonzalo, Rui Gusmao and Angela Dago for their faithful and continuous technical assistance.

In addition, the generous support and contribution of all my colleagues, friends and families are deeply acknowledged and emphasized in all cases of my future life.

INDEX

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS AND ABBREVIATIONS

AdCSV	Adsorptive Cathodic Stripping Voltammetry
ASV	Anodic Stripping Voltammetry
BiFE	Bismuth Film Electrode
BiSPCFE	Bismuth Modified Screen Printed Carbone Electrode
BLM	Biotic Ligand Model
CCSCP	Constant Current Stripping Chronopotentiometry Potentiometry
CV	Cyclic Voltammetry
DME	Dropping Mercury Electrode
DPV	Differential Pulse Voltammetry
ESI-MS	Electron Spry Ionization Mass Spectrometry
FIAM	Free Ion Activity Model
GSH	Glutathione
HMDE	Hanging Mercury Drop Electrode
ICT	Isothermal Calorimetry Titration
MCR-ALS	Multivariate Curve Resolution Alternating Least Square
MFE	Mercury Film Electrode
MTs	Metallothioneins
NMR	Nuclear Magnatic Resonance
PC_n	Phytochelatins
RSD	Relative Standard Deviation
SCE	Size-exclusion Chromatography
SPCE	Screen Printed Carbone Electrode
SVD	Singular Value Decomposition

ABSTRACT

The application of Bismuth modified cheap and disposable screen printed carbon electrode (BiSPCE) for voltammetric studies of metal–thiol rich peptides complexation was evaluated considering systems consisting of cadmium as a metal and GSH and PC_2 as thiol peptides. Comparison of the performance of BiSPCE with the commonly used glassy carbon electrode (BiFE) was made. The information obtained about the complexation sequence using the former electrode was quite consistent with previous studies made on GSH and Cd systems using the conventional mercury electrodes due to the absence of signal splitting, the good sensitivity and the wider linearity range. In contrast with the conventional mercury electrodes, the anodic signals associated with bismuth electrode material were observed to be weakened and all the available signals were well resolved which shows the suitability of Bismuth based electrode for metal- thiol complexation studies. MCR-ALS could not be applied due to the continuous shift of the peak potentials, loss of linearity of the species and anomalous shape of the complex signal formed between Cd and GSH. Therefore, the complexation sequence between GSH and Cd^{2+} was evaluated qualitatively. However, a relatively well defined shape and intense voltammograms were observed for Cd-PC_2 system and consequently, MCR-ALS was applied after correction of the continuous peak potential shift for the complex signals. The peak intensity associated with complex signal was dominated by the intense signal of the free metal reduction in acidic and fairly basic medium. However a relatively intense signal of the complexes was obtained at pH 7.5 in borate buffer solution. In addition extensive Bi complexation was observed from ESI-MS experiment which proves the suitability of ex situ mode of Bi film preparation for thiol- metal complexation studies by voltammetric titration technique.

Key words: Differential pulse voltammetry, Bismuth film electrodes, Screen printed carbon electrode, ESI-MS, MCR-ALS.

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Key words: Differential pulse voltammetry, Bismuth film electrodes, Screen printed carbon electrode, ESI-MS, MCR-ALS.

INTRODUCTION

1. INTRODUCTION

1.1 Toxicity and Sources of Cadmium

The presence of toxic metals such as Cd and Pb in the environment has been a source of concern to environmentalist and governmental agencies. This is mainly due to their health implication since they are toxic above a certain tolerable level to human, animals and plants. The adverse effect of these metals is aggravated by their ability to accumulate in the living organisms and resist for bio-degradation process. Although the existence of cadmium containing carbonic anhydrase from some marine diatoms has been recently found [1], it is generally accepted that Cd^{2+} is non-essential metal for both plants and animals. The International Agency for Research on Cancer has classified cadmium as human carcinogen [2]. In plants Cd^{2+} causes various effects, such as inhibition of photosynthesis, respiration, nitrogen metabolism as well as the decrease of water and mineral uptake [3]. Those Cd^{2+} - induced changes in plant metabolism finally lead to inhibition of plant growth. Cd^{2+} inhibits the growth or production of cells in mammals, causes cell death and may cause cancer [4]. Particularly in human it replaces the role of Zn in enzymes and accumulates in the bones, kidney, and liver leading to failure after prolonged exposure [5]. Although the final reaction of plants and mammals to Cd^{2+} may be different, many Cd^{2+} related processes are common in both types of cells [6].

Cadmium is naturally present in the environment as minor constituent of soil, surface and groundwater as hydrated ion and inorganic complexes such as carbonates, hydroxides, chlorides or sulphates, or as organic complexes with humic acids [7]. However, a wide range of human activities contributed to the current increased level of Cd metal in the natural environment. Cadmium is commonly introduced to the natural environmental systems through atmospheric deposition, direct discharge from industrial operations, leakage from landfills and contaminated sites, and the use of sludge and fertilisers in agricultural activities.

1.2 Effect of Cadmium Speciation on its Toxicity

Generally, significant relationship have been found in many cases, between metal bioavailability and free metal ion concentrations as predicted by the Free Ion Activity Model (FIAM) [8] or the Biotic Ligand Model (BLM) [9]. It should be noticed that the free metal ion activity or bioavailability in the natural environment is not a function of only the total recoverable amount of metal rather, speciation of the metal in the medium greatly affect the bioavailability of the metal. The concept of "toxicity is simply a function of the total concentration of a metal" can be or extremely oversimplification of the truth in some cases. Nowadays, the importance of considering bioavailability in assessing ecological impacts of metals has been recognized by both regulatory authorities and the scientific community in contrast to the original concept which relates heavy metals toxicity solely with the total recoverable amount [10]. More specifically various studies on the speciation have shown that absorption of toxic metals like cadmium by an organism decreases in the presence of natural organic and inorganic ligands due to the complexation phenomena [11, 12, 13, 14] according to :

$$M + mL \rightleftharpoons ML \ldots ML_m \quad (1)$$

Phytochelatins (PCs) produced by plants and Metallothioneins (MTs) synthesized by animals are among those essential naturally occurring metal-binding organic ligands that play a key role in metal binding processes and thus influencing metal speciation, bioavailability and toxic effect in cellular environments [15]. In the particular case of metal detoxification (i.e., the response of the organism to an excessive uptake of heavy metals), the process involves simple molecules like glutathione and longer chain peptides MT and PCs. PCs and MTs are different classes of cysteine-rich, heavy metal-binding protein molecules. PCs are enzymatically synthesized peptides, whereas MTs are gene-encoded polypeptides [16]. The thiol groups of MT and PCn ensure the formation of very stable metal-peptide bonds which immobilize the metal, thus decreasing its toxicity and making its elimination easier.

Currently plant derived phytochelatins are being used for developing a technology called Phytoremediation that can potentially reduces the problem of contaminated soil or water with

toxic metals [18]. In this technology genetically engineered plants are involved that uptake metal and metalloids ions from the polluted site by the process called phytoextraction. Therefore it is quite interesting to study the complexation mechanism of metals with plant thiols for better understanding of the role of phytochelatins and the mechanism in metal detoxification processes.

1.3 General Description of Phytochelatines (PCn)

PCn were first identified in the fission yeast *Schizosaccharomyces pombe*, currently PCn have been found in some fungi, some marine diatoms, and all types of plant species investigated [4]. They act as high affinity metal chelators and for this reason plant cells are capable of tolerating high levels of metals like Cd^{2+}. It was well confirmed that metal induced phytochelatin production decreases cellular levels of glutathione [16]. Hence they are synthesized from a precursor glutathione in the presence of some metals such as Cd, Cu, Hg, As or Pb, in a reaction controlled by enzyme *γ- glutamylcysteine dipeptidyl transpeptidase (PC synthase)* that catalyzes the transfer of γ- glutamylcysteine dipeptide part of GSH to an active GSH molecule for growing of PC chain according to the following reaction [18]:

$$\gamma\text{-Glue-Cys-Gly} + (\gamma\text{-Glu-Cys})_n\text{-Gly} \longrightarrow (\gamma\text{-Glu-Cys})_{n+1}\text{-Gly} + \text{Gly} \qquad (2)$$

The above reaction is highly dependent on the presence of heavy metal in the plant cell and it has been shown that the efficiency of activation of the metals on the enzyme is according to the following:

$$Cd^{2+} > Ag^{+} > Pb^{2+} > Cu^{2+} > Hg^{2+} > Zn^{2+} > Sn^{2+} > Au^{3+} > As^{5-} > In^{3+} > Tl^{3+} > Ge^{4+} > Bi^{3+} > Ga^{3+}$$

However 39 elements in the periodic table have no any tendency to activate the enzyme responsible for PC synthesis [18].

PCn are represented by a formula of $[\gamma\text{-Glu}(\text{-Cys})]_n\text{-Glyc}$ where n varies from 2 to 11 and their general structure is depicted in figure 1 .

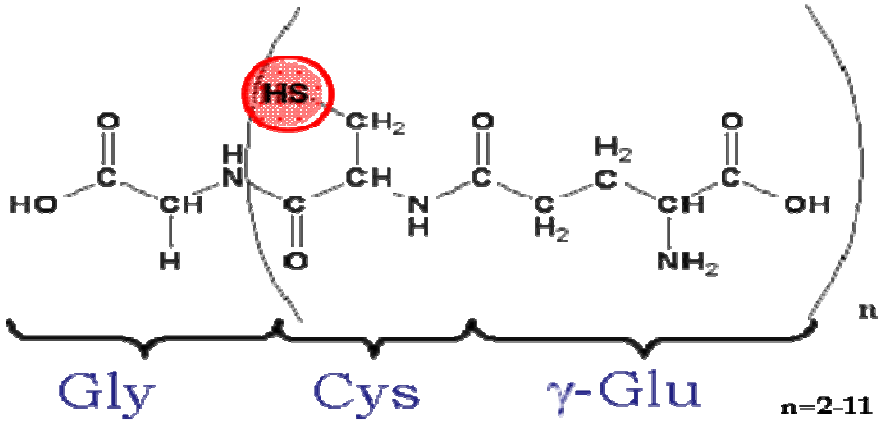

Figure 1. The general structure of phytochelatins

More than 90% of toxic metal like Cd^{2+} interring the cytosol of a given plant cell is accumulated as Cd-PC complexes temporally in the plant membrane organelle which is called vacuole (Figure 2 b). To summarize the process the metal ions activate the latent PC synthase to produce PC from GSH, and the PC molecules of various chain length form PC-metal complex. Then the PC-metal complexes are transported to the vacuole and this transport is energized by hydrolysis of ATP [18]. The process is summarized in figure 4.

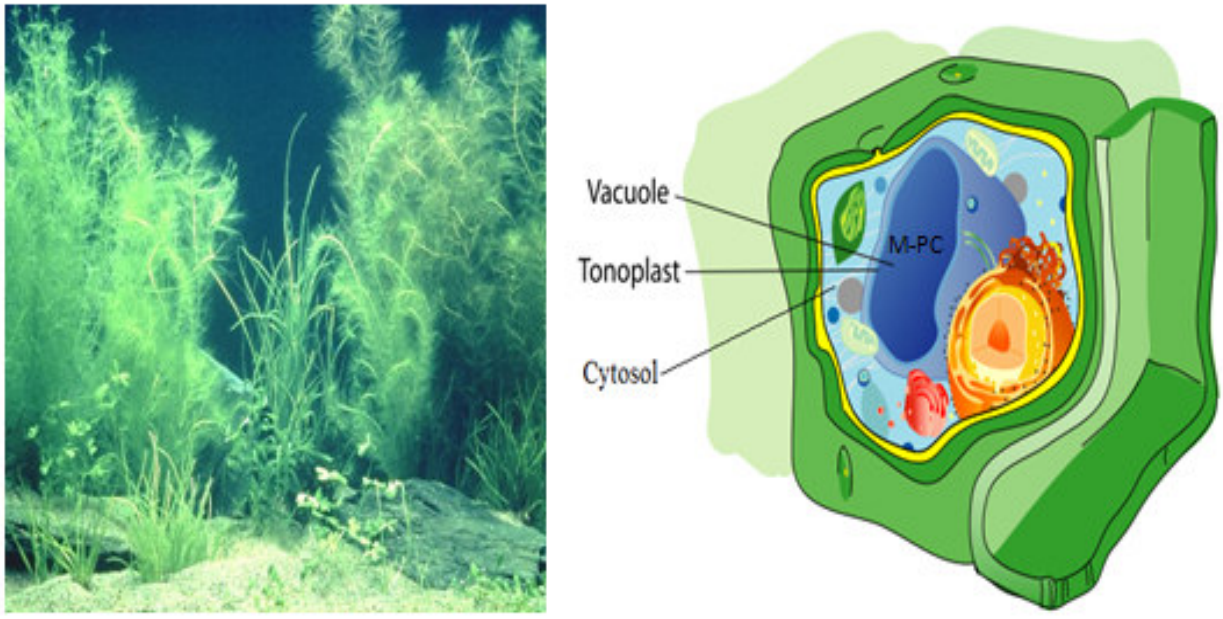

Figure 2. (a) A typical plant capable of synthesising PC (b) Plant cell structure showing the place where PC-metal complexes are stored

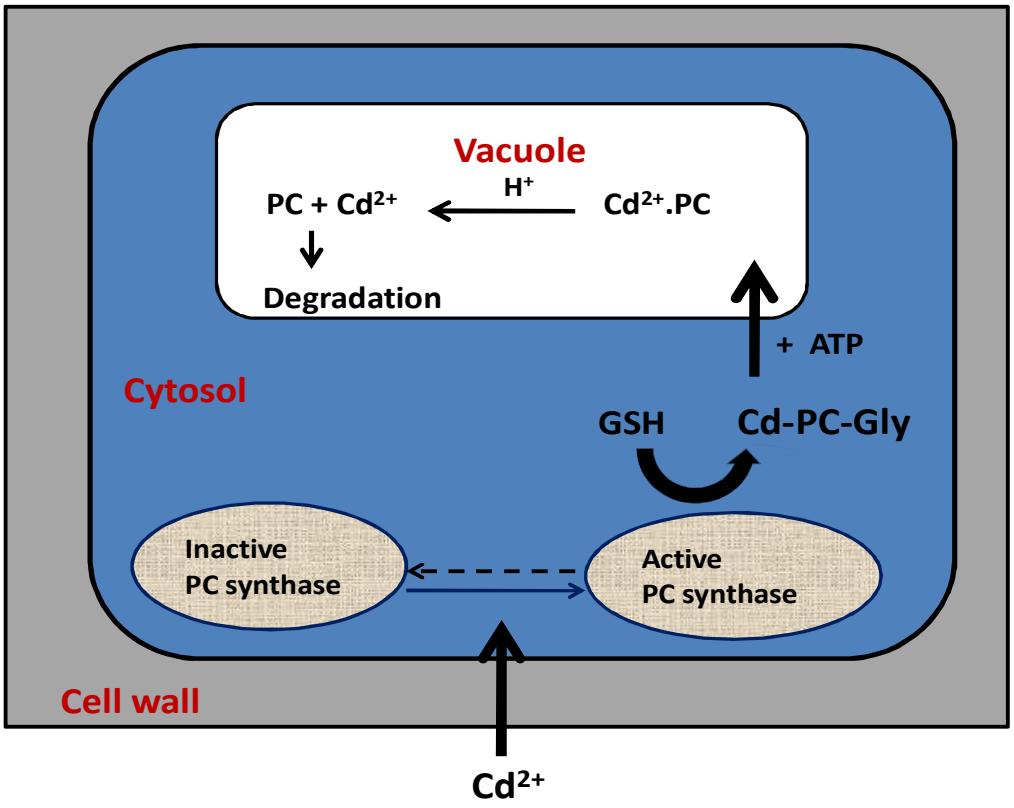

Figure 3. Systematic representation of the process of detoxification of Cd^{2+} by PC in a plant cell

Although animal metallothioneins are relatively well characterized and confirmed to contain cysteinyl-sulphur coordinated metal ions arranged in multinuclear center (clusters) the structural information about phytochelanies metal complex is not fully understood yet [19]. However the relative stability and the possible stoichiometries of the resulting complexes formed were extensively studied by various techniques.

1.4 Analytical Methodolegies for Characterization of Metal-thiol Peptide Complexation

The speciation of toxic metals in the presence of phytochelatins and essential biological compounds have been studied by various techniques extensively. An approach based on size-exclusion chromatography (SEC) with off-line mode for the detection of phytochelatins and atomic absorption spectrometry for metal quantification has been used to study Cd and Pb

phytochelatin complexes distribution [20]. In the study of Cd^{2+} complexation with GSH in human erythrocytes glutathione was monitored by ^{1}H-NMR [21]. UV spectroscopy and potentiometric techniques were also applied for quantification of free glutathione and free cadmium ion respectively to explain the mechanism of cadmium complexation with gluthation [22]. More recently Isothermal Titration calorimetry (ITC), ESI-MS and electroanalytical techniques were used simultanously for study of competitive Binding of Cd and Zn with the Phytochelatin (γ-Glu-Cys)$_4$-Gly [23].

However, electroanalytical techniques are one of the imperative ways for metal speciation studies because of their low detection limit, high selectivity for a particular redox state of a species, speed, low cost and convenient operation. In addition they can be employed for field (at-site) study and flow analysis methodologies [24]. Particularly voltammetric and chronopotentiomeric techniques have been extensively used to study the metal complexation phenomena with phytochelatins and other biological organic ligand using mercury electrodes and other material film electrodes . The common way of characterization of the complexation process is by titrating the metals with the ligand or using the reverse process to get a progressively changing ligand-to-metal ratio that yield valuable information about the relative stability of the metal bound in different manners and possible stoichiometries of the resulting complexes.

Developing electroanalytical methods having sufficient detection limit down to the cellular level is essential to model the complexation process in the living organism cell. To achieve that, anodic stripping techniques seems to be a prior option. However, due to the strong attachment of the metal ion on to the thiol site of the ligand, oxidative striping of the metal from the complex is not possible because the reduction of the metal complex becomes irreversible [25]. For this reason adsorption stripping chronopotentiometric (AdSCP) method, which is less affected by the presence of organic matter, and performs a reductive stripping of the previously adsorbed complex, was proposed for the study of thiol-metal complexation [26]. However differential pulse polarography or voltammetry are being used extensively to study complexation because of their low detection limit which are still enough to quantify low level metal and ligand concentration comparable to those existing in organisms cells and they present the advantage of a

higher simplicity. Moreover the appearance of signals for the free peptide, free metal ion and complex in different peak potentials position makes this technique to be more preferable.

1.5 Types of Electrochemical Measuring Systems for Thiol peptides-Metal Complexation Studies

1.5.1 Electrochemical System Based on Mercury Electrodes

Mercury electrodes, most frequently the mercury film electrode (MFE), the hanging mercury drop electrode (HMDE) and the dropping mercury electrode (DME) have been used extensively for routine stripping analysis and complexation studies. This is due to some important advantages over solid electrodes and other material film electrodes that are used for anodic scanning purposes. First, mercury is the best for cathodic scanning because of its large hydrogen over voltage that extends the negative limit of potential window and show a well defined electrochemical behavior in the negative potential region with very low background and noise as compared to the solid electrodes [27]. The other advantage originated from the periodic renewal of the surface of the electrode that reduces the problem of contamination. However, the main disadvantage of this electrode and the corresponding film electrode (MFEs) is related to its toxicity, volatility, and disposal and so that it is considered environmentally undesirable [28]. In addition to this as mercury electrodes have low mechanical stability and require special handling their application for flow analysis is very limited [28].

With regard to the application of mercury for the study of thiol- metal complexation, its good sensitivity and reproducibility for free metal, free ligand and complex entities facilitates such studies by polarographic techniques. However, mercury electrodes usually give complicated sets of overlapping signals that correspond to the reversible reduction of metal ions, the non reversible reduction of strongly bound metal to the thiol peptide and signals associated with the oxidation of mercury electrode itself [23, 25, 26]. In the latter case the oxidized mercury forms thiol complexes by itself to give important anodic signals which complicate the interpretation of the complexation process. This makes necessary the use of chemometric techniques, especially

multivariate curve resolution by alternating least squares (MCR-ALS) to resolve the signals of pure species and the evolution of their concentrations during the titration.

Due to the aforementioned disadvantages of mercury electrodes different inert solid materials like carbon, silver, and gold electrodes have been tried as a substituent of mercury electrode [29]. These electrodes exhibit good mechanical stabilities together with the possibilities for surface modification which increases their performance. However, bare solid electrodes suffer from memory effects due to lack of surface regeneration and consequently, either polishing or a tedious chemical or electrochemical surface regeneration is frequently required [28]. As a result electrodes which are environmentally friendly and that have properties which removes the problem associated with the use of solid electrodes are being sought continuously. For the first time Wang and coworkers introduced an environmentally friendly bismuth- film electrode by their pioneered works as a best alternative electrode for mercury and bare solid electrodes [30].

1.5.2 Electrochemical System Based on Bismuth Film Electrodes

Researches within the past few years have shown that the performance of bismuth film electrodes (BiFE) which is composed of metallic bismuth on a conductive supporting substrate is comparable or even surpass that of the conventional mercury electrodes that are being used commonly for anodic stripping voltammetry analysis [31] and chronopotentiometric stripping analysis [32]. In addition, adsorptive cathodic stripping voltammetry (AdCSV) has been successfully employed at Bi film electrode [33]. The most important characteristics of this electrode are simplicity of preparation, high sensitivity , good mechanical stability , well defined and highly reproducible stripping signal, good resolution between neighboring peaks, low background characteristics , less affected by potential interferents such as surfactants and ionic species, large cathodic potential working range, removal of the film easily when it is required and being insensitive for dissolved oxygen that greatly reduce the time required and amount of nitrogen used for the deoxygenation step [33]. The high sensitivity of the electrode is due to the property of Bismuth to form fused alloys with heavy metals, which is analogous to the amalgams that mercury forms according to equation 3. In addition, bismuth is known to be a non-toxic

element [35] as compared to mercury electrode whose use as an electrode material may in the future be constrained.

$$M^{n+} + ne^- \rightleftharpoons Bi(M^0) \quad (3)$$

Specifically more recently Bismuth modified glassy carbon electrode (BiFE) was proposed as an alternative method for complexation study [34]. It was shown that all signal observed in mercury film electrode were also present in voltammograms obtained using Bismuth film electrode. Moreover, good improvement was observed towards the problem associated with signal overlapping. However it was noticed that the reduction process of the complexes showed remarkably inert electrochemical character on bismuth film electrodes that hinder to do experiments at very low concentration (lower than $2x10^{-5}$ M) that were easily achieved by using mercury film electrodes. In addition the free ligand evolution was not linear for concentration higher than $2x10^{-5}$ M and its shape changes with increasing concentration consequently it was not possible to apply MCR-ALS analysis. Fortunately this did not prevent qualitative interpretation of the complexation process as all the signals were well resolved in contrast to the signals observed using mercury film electrode. In light of the above mentioned thiol-complexation study using BiFE, in this study the applicability of cheap and disposable screen printed carbon electrode modified with bismuth was tested for heavy metal complexation with phytochelatines considering the case of cadmium complexation with gluthation and PC_2 as a model ligand for the higher thiol peptides and other organic ligands.

1.6 Screen Printed Carbon Electrodes

Disposable carbon electrodes have a typical complete electrochemical cell configuration, i.e they combine the working, reference and auxiliary electrode together and are highly suitable for working with micro volumes and decentralized assays or to develop specific sensors by modifying their surface with various materials. The typical screen printed carbon electrode by Dropsens (Oviedo, Spain) is based on *Ceramic substrate*: H33 x L10 x W 0.5 mm, *Electric contacts:* Silver. The electrochemical cell consists of *Working electrode*: Carbon (4 mm

diameter), *Counter electrode*: Carbon and *Reference electrode*: Silver. The arrangements of the parts of this electrode are shown in Figure 2.

The screen printed electrode is connected to any model of potentiostate by a special type of connector which is manufactured by Dropsens that acts as bridge between the screen-printed electrode and potentiostat. Figure 2 shows the two kinds of connector used for screen printed electrodes. The sample is usually applied on the working electrode part in the form of small drop on isitu or in the laboratory for stripping analysis of environmental or biological samples.

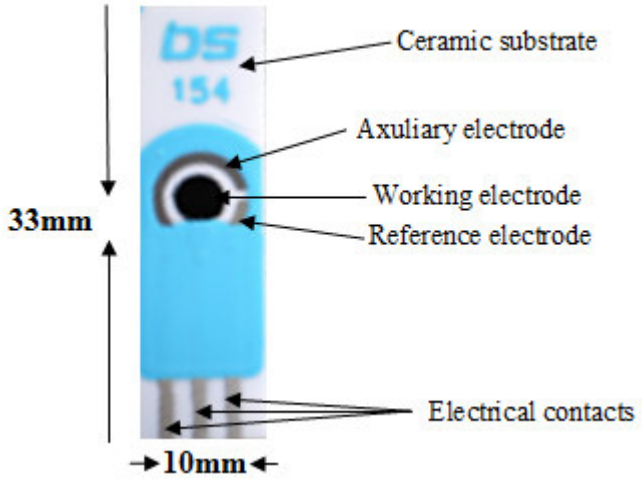

Figure 4. Typical Dropsense screen printed electrode

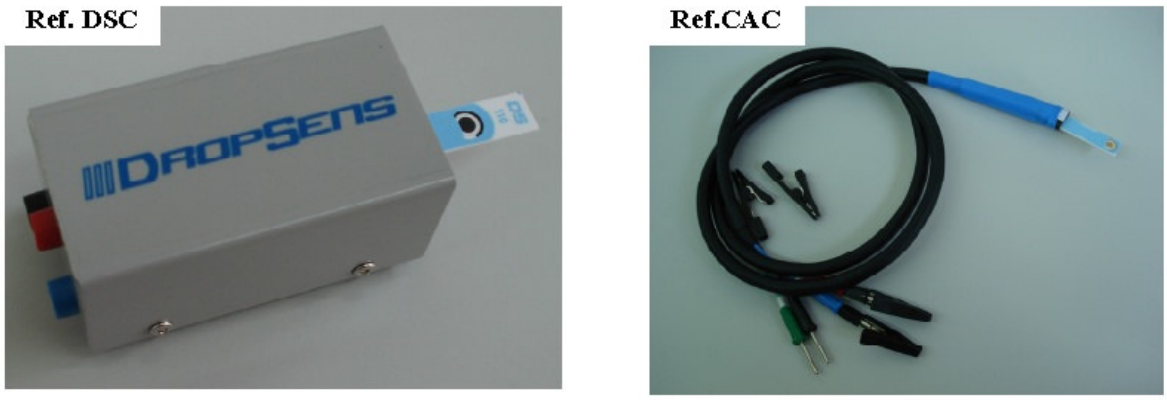

Figure5. The two common types of connector that links screen printed electrodes to the potentiostat (taken from Dropsens)

In addition to the above mode of application disposable carbon electrodes can also be used as working electrode in the place of the conventional working electrodes in an electrochemical system. The most important advantages of disposable screen printed electrodes are their inexpensive price, disposable character, flexibility in design and that they are easy to produce. For this reason one can use a new screen printed electrode every time, which eliminate the problem associated with the carry over contamination and reutilization and reduce the fear of expensive damage associated with reusable electrodes. In addition the working surface of solid electrodes including glassy carbon usually needs to be polished quite frequently before formation of bismuth film on them which leads to difficulty to use such electrodes with high precision for online analysis with the use of automated systems and for on-site filed portable instruments [29]. For this reason screen printed electrodes (SPEs) can be a good substrate for preparation of film electrodes for the purpose of anodic stripping voltammetry measurements of trace elements and speciation studies. Another important advantage of the screen printed carbon electrode provide a better surface structure for platting of bismuth as the film electrode on SCPCE showed a better sensitivity in stripping analysis than the corresponding glassy carbon electrode [28]. In this study the carbon screen printed electrode modified with bismuth film was used as working electrode for thiol–metal complexation studies.

1.7 Overview of Modification of Surface of Electrode by Bismuth

Bismuth can be plated on several materials, such as glassy carbon [30], carbon paste [36], Screen–printed carbon ink [37], carbon- fibers [38] and gold [39]. A large number of studies used carbon electrodes as supporting material for preparation of Bi-film electrode. The common way of preparation of BiFEs is by electroplating of the conductive supporting material such as glassy carbon, carbon pest, and screen printed carbon ink electrode with Bi from Bi(III) solution by applying a negative potential in the reductive potential regime at either in situ or ex situ condition. In addition the surface of those electrodes can be modified with Bi_2O_3 to further electrochemically reduce bismuth

1.7.1 Ex situ plating

Ex situ mode of film electrode preparation involves electroplating of the substrate material with the Bismuth from a solution containing Bi (III) ion separately and before the use of the electrode for analysis in the pre-analysis step. Consequently this requires a transfer of the prepared bismuth film electrode to the measurement solution or in flow system. Since bismuth film prepared is used for several measurements the overall stability of the electrode is very important [40]. It has been reported that formation of mercury film electrode by ex situ mode is complicated by the difficulties associated with the preparation of the mercury coating which result in the loss of mercury up on transfer to a solution to be measured [41]. In contrast, stability problem with bismuth film electrode prepared using normal sized glassy carbon electrode or screen carbon electrode were not observed [42]. The ex situ mode of deposition of bismuth film was also proved to be a better option of film electrode preparation by a previous study for study of metal complexation where the presence of Bi(III)-ions in the solution could interfere with the speciation of other metals [43].

1.7.2 In situ plating

The Bi film electrode can be electrochemically formed by in situ mode if the film is plated on the substrate material simultaneously with the target metal ion to form metal alloy during the preconcentration step from the solution containing about 400 to 1000 µg/L Bi(III) in stripping analysis. As in situ plating does not require separate bismuth plating the time required for completion of the analysis is shortened and the stability of the film is not a critical problem. However, in situ plating is limited for fairly acidic pH range of the sample solution as the Bi(III) ion undergoes immediate hydrolysis at natural and basic pH condition.

$$Bi^{3+} + 3H_2O \longrightarrow Bi(OH)_3 + 3H^+ \quad (4)$$

However, interestingly in situ plating can be applied at extremely high pH values and this is because instead of insoluble $Bi(OH)_3$ soluble and stable complex is formed according to the following reaction:

$$Bi^{3+} + OH^- \longrightarrow Bi(OH)^{2+} \quad (5)$$

During the deposition process the complex ion is reduced on the electrode surface as the free Bi(III). The above property is one of the advantages of BiFE over MFE as the later is known to be non functional at extreme basic conditions [28].

1.7.3 Bi_2O_3 Modified Electrodes

BiFE is also prepared by modifying the surface of the substrate electrode with Bi_2O_3 [44]. This is based on the reduction of Bi_2O_3 on the surface electrode by applying a sufficiently high negative potential, usually -1.0 V vs Ag/AgCl according to the following reaction:

$$Bi_2O_3(s) + 3H_2O + 6e^- \longrightarrow 2Bi(s) + 6OH^- \quad (6)$$

Then the electrode is said to be activated and can be used readily for the intended purpose. It is interesting to note that this mode of BiFE preparation does not require Bi(III) for deposition of Bi film and can be applied simply for in situ analysis after activating at an appropriate potential. However Bi_2O_3 is prepared by mixing it in carbon paste to integrate it into the electrode surface; therefore this mode of BiFE preparation is practically confined to carbon paste electrodes.

1.8 Objectives and Scopes

1.8.1 General objectives

This work reports the application of a new method for complexation studies of cadmium with phytochelatins using screen printed carbon electrodes which are modified by plating with

bismuth in ex situ mode of deposition (BiSPCE). Although cadmium phytochelatin complexation has been extensively studied by polarographic techniques and other analytical methods the current study evaluate if there are significant advantages of using BiSPCE in resolution of overlapped signals, and minimization of the number of signals in order to facilitate the interpretation of the process. Also the possibilities of application of MCR-ALS will be evaluated for this particular system which was not possible in the case of BiFE.

1.8.2 Specific objectives

- To develop a method for the study of cadmium complexation with gluthatione and PC_2 using bismuth modified screen printed carbon electrode (BiSPCE) with special attention to the optimization of the experimental conditions.
- To compare critically the results obtained from BiSPCE with those results from conventional glassy carbon disk electrode (BiFE) and mercury film electrodes (MFE) and to see the possible merits of bismuth modified screen printed carbon electrodes over that of the MFE and BiFE.
- To apply MCR-ALS methodologies to resolve the concentration profile and the pure signal of each species during the titration process
- To propose possible complexation mechanisms and compare the results with other literature results and the result obtained from ESI-MS experiments.
- Apply ESI-MS experiments to understand the different behaviour of the anodic signal of thiol compound in bismuth film electrodes.

EXPERIMENTAL

EXPERIMENTAL

2.1 Apparatus

All voltammetric measurements were performed using a Metrohm 663 VA Stand (Metrohm, Switzerland) interfaced to a computer controlled potentiostat/galvanostat Autolab System PGSTAT12 (Eco Chemie, The Netherlands) and measurements were controlled by a general purpose electrochemical software operating system, Autolab GPES 4.9 (software version for windows 2000 and XP). Bi modified glassy carbon electrode (BiFE) of 2mm diameter (Metrohm) and Bismuth modified Carbon Screen Printed Electrode (BiSCPE) 4 mm diameter provided by Dropsens (Oviedo, Spain) were used as working electrodes. The reference electrode and the auxiliary electrode were Ag/AgCl/KCl (3 mol L^{-1}) and a Pt wire, respectively. The whole electrochemical set up is shown in Figure 6 and Figure 7.

Figure 6. A photograph showing the electrochemical system used in this study

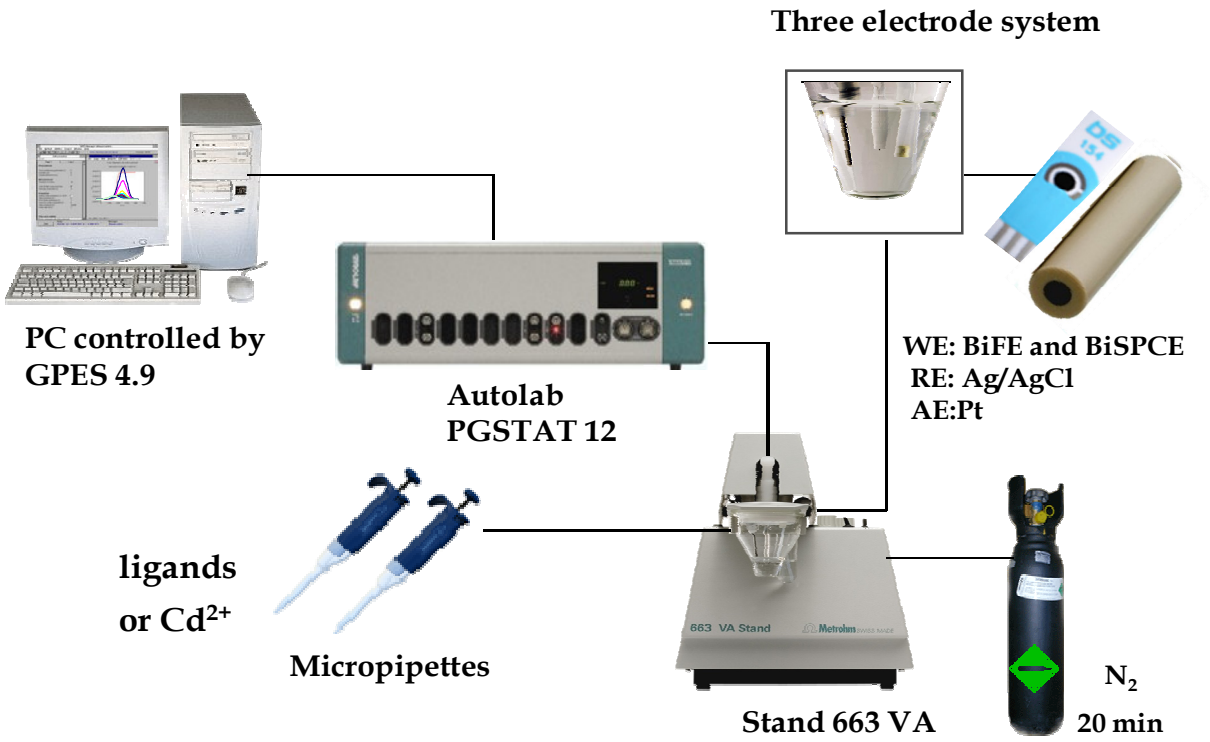

Figure7. Flow chart for the electrochemical set up

The electrochemical parameters used for DPV and DPASV measurements were a pulse time of 50 ms, pulse amplitude of 50 mV, potential step of 2 mV. During DPASV measurement the deposition potential (Ed) was applied for 60 s and a rest period of 5 s was made between the deposition and the stripping steps. For cyclic voltammetery measurement the scan rate was set at 0.1 V/s.

The measurement of pH value during the experiments was carried out by means of a Crison micro pH meter. All experiments were performed at a controlled room temperature of 20^{o}C.

ESI-MS experiments in positive ion-mode for a mixture containing Cd(II), B(III), GSH and PC_2 were done using an Agilent 1100 Q-TOF instrument. The instrument control was performed using Analyst QS software.

2.2 Reagents

Glutathione (GSH), in the reduced form, was provided by Merck with purity greater than 99% and Phytochelatins with n = 2 was provided as trifluoroacetate salt by Diver- Drugs S.L. (Barcelona, Spain) with a purity of *ca*. 90% . A standard Bi(III) solution (996 gL^{-1}, atomic absorption standard solution) was purchased from Fluka. All other reagents used were Merck and Sigma/Aldrich analytical grade. 10^{-2} mol L^{-1} Cd(II) stock solutions were prepared from $Cd(NO_3)_2 \cdot 4H_2O$ and standardized complexometrically. Borate buffer were prepared from sodium tetraborate, and the pH was adjusted to the value of 7.0, 7.5 and 8.5 with the addition of ultra pure HNO_3 (65%, Merck). Maleic acid–KOH buffer solution was used for pH control at the value of 6.4. KNO_3 was employed as supporting electrolyte. Ultrapure water (Milli-Q plus 18.2 systems, Millipore) was used in all experiments.

1.1 Preparation of Film Electrodes

2.3.1 Preparation of Bismuth Modified Glassy Carbon Electrode

New BiFE were prepared every day immediately before voltammetric measurements. Ex situ preparation of the Bi film on glassy carbon electrode was performed based on an optimized procedure as described in N. Serrano *et al* [17]. To describe it briefly, prior to bismusth film formation the glassy carbon electrode which serves as the supporting substrate was properly polished with 0.05 μm alumina powder suspension on a polishing pad (metrohm) and washed several times with ultrapure water and sonicated for several minute in ultrapure water bath . Then the electrode was rinsed with pure water and pure alcohol and connected to the electrochemical system as working electrode and placed in a plating solution containing 100 ppm Bi(III) solution in 0.2 M acetate buffer (pH=4.5). After deoxygenation of the solution for 20 min with pure nitrogen the bismuth film was generated using a deposition potential of -0.6V applied for 300 s with stirring at the rate of 500 rpm. The solution was left for 20 s without stirring to equilibrate. After preparation of BiFE all electrodes of the electrochemical system were washed several times with ultrapure water carefully without scratching the bismuth film. Finally the Bi(III) solution was substituted with the analyte solution to be measured.

2.3.2 Preparation of Bismuth Modified Screen Printed Carbon Electrodes

To prepare BiSPCE the same procedure was applied as BiFE preparation with slight modification. To describe it briefly, first the screen printed carbon electrode was connected to the potentiostat as working electrode using a 1 meter length cable provided by the electrode manufacturer (Dropsens). Then the three electrode system was immersed to a 20 mL solution containing 100 ppm Bi (III) in 0.2 M acetate buffer at pH 4.5. The solution was deoxygenated with pure nitrogen for 20 min followed by deposition of bismuth on to the screen printed electrode at deposition potential of -0.8 V for 300 second with solution stirring (by means of an external mechanical stirrer) and then the solution was left for 20 s without stirring to equilibrate. Finally, before using voltammetric measurements of the analytes solution the whole electrode system was rinsed with ultrapure water several times without scratching the bismuth film on the screen printed electrode.

2.4 Procedure for Voltammetric Measurements

2.4.1 Cyclic and Differential Pulse Anodic Striping Voltammetry Measurements

In order to assess the useful potential window and reversibility of electrochemical reactions on the BiSPCE cyclic voltammetry measurements were done at different pH values. CV of borate buffer at pH 7.0, 7.5, 8.5 and maleic/maleate buffer at pH 6.4 were measured in the absence of either peptide ligand or Cd(II) to select the widest potential window. 20 ml of the respective buffer solution were placed in to the voltammetric cell and the solution was deoxygenated for 20 min then the CV was recorded for each buffer system at scan rate of 0.1 V/s with the limit of first and second vertex potential at -0.5 and -1.6 V respectively. In addition differential pulse anodic striping voltammetry was recorded for Cd(II) solution to locate the peak potential at the selected pH value (7.5) by placing 20 mL of borate buffer solution in to the voltammetric cell and 40 µL of 10^{-2} M Cd(II) solution followed by purging for 20 min then DPASV was recorded. In addition, before each voltammetric titration process, the presence of Cd(II) was checked by recording DPASV of the blank buffer solution.

2.4.2 Adsorptive Cathodic Stripping Voltammetery Measurements

The procedure used to obtain adsorptive cathodic stripping voltammograms was as follows: 20 mL of 0.05 M borate buffer solution at pH 7.5 was transferred in to the voltammetric cell. The stirrer was switched on and the solution was purged with nitrogen gas for 20 min. Then accumulation was effected for 120 s at -0.6 V whilst stirring the solution. At the end of the accumulation time the stirrer was switched off. After 5 s had elapsed to allow the solution to become quiescent, the potential was scanned from -0.6 to -1.1 V using differential stripping voltammetry. When further ligand or metal solution was added to the cell, the solution was deoxygenated with nitrogen before carrying out further voltammetric measurement.

2.4.3 Differential Pulse Voltammetric Measurements

Differential pulse voltammetry was performed according to the following procedure: 20 ml of buffer solution containing 0.01 M maleic–maleate (pH=6.4) and 0.05 M KNO_3 as supporting electrolyte or a buffer solution containing 0.05 M Borate (pH = 7.5, 7.0 and 8.5) were put in an acid cleaned and dried glass cell. Then the solution was purged with pure nitrogen for 30 min and blank anodic stripping voltamograms were recorded to check the presence of cadmium contamination in the solution. Then to investigate the voltammetric nature of pure Cd^{2+} and GSH, solutions containing these pure species were added to the cell to obtain concentration range of 10^{-4} to 10^{-7} M in the voltammetric cell and the respective voltammograms were recorded. Titration of Cd^{2+} with GSH or PC_2 was done by adding 40 µL of $1x10^{-2}$ M Cd^{2+} solution to the cell to get its concentration exactly $2x10^{-5}$ M and titrating it with $1x10^{-3}$ M freshly prepared GSH or PC_2 solution in order to get various ligand to metal ratios (from 0 to 3). The reverse titration was also done in such a way with successive addition of $1x10^{-3}$ M Cd^{2+} solution to $2x10^{-5}$ M GSH solution in the voltammetric cell. Voltammograms were measured after deoxygenation of the solution for 1 min with stirring after each addition of GSH or Cd^{2+} solution to the cell.

2.5 ESI-MS Experiments

The ESI-MS with positive mode was performed for a mixture containing GSH, PC_2, Bi^{3+}, and Cd^{2+} in Ammonium Acetate/Ammonium Hydroxide Buffer at pH 7.5 and using mobile phase made of 5 mM ammonium acetate in water–acetonitrile mixture (90:10, v/v) at pH 7.5.

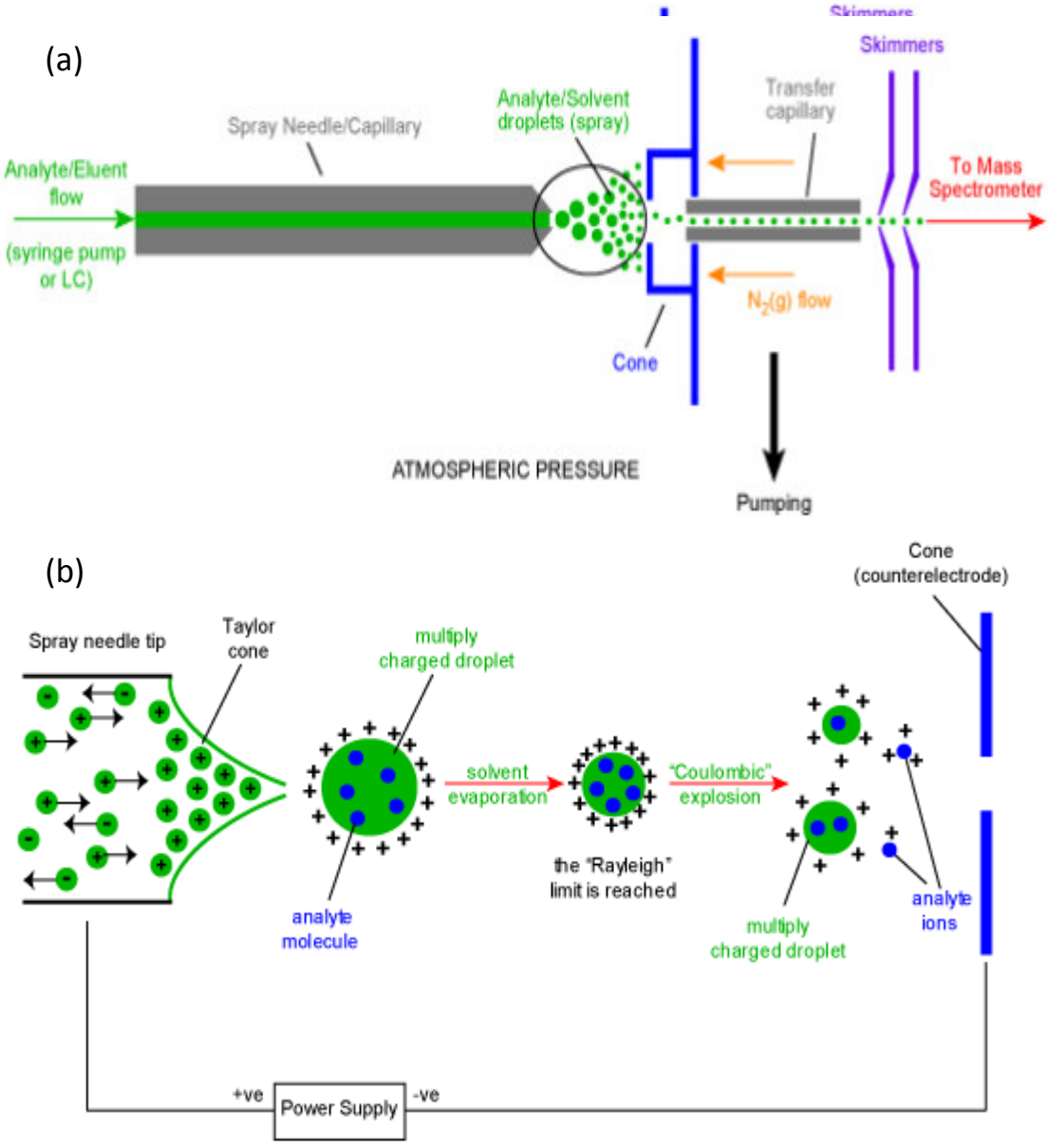

Figure8. (a) Schematic representation of the ESI-MS ion source (b) A schematic of the mechanism of ion formation in ESI-MS.

The operational procedure was adapted from E.Chekmeneva *et.al* [45]. To explain briefly: samples were introduced in to the electron spray source by direct injection mode of 50µL in Ammonium Acetate/Ammonium Hydroxide Buffer at pH 7.5 at flow rate of 40 µL /min at a source temperature of 300 ^{o}C. The applied voltage was maintained at 4.0 kV for the capillary and 200 V for the fragmentor. The mass spectra were collected through m/z range from 100 to 1500. The mixture containing GSH, Bi^{3+} and Cd^{2+} were prepared by mixing $1x10^{-3}$ M of each species in the buffer solution to get ratio of Cd^{2+}: GSH and Bi^{3+}:GSH (1:1:1, 1:2:1, 2:1:1) and Cd^{2+}:Bi^{3+}:GSH(1:1:1, 1:1:2, 2:2:1, 1:2:1, 2:1:1, 1:1:4).

ESI-MS is especially useful in producing ions from macromolecules where the analyte often requires that non-covalent molecule-protein or protein- metal complexes are representatively transferred into the gas-phase by overcoming the propensity of these molecules to fragment when ionized. This is the reason why this technique was selected for this particular investigation as it helps to see unfragmented PC-Metal complex in the spectra. Figure 8 summarises the main steps in ion production in ESI-MS technique.

As any other mass spectroscopic devices ESI-MS combines four basic parts. Ion source, mass analyzer, detector and recorder. As stated above the unoqe properties of ESI-MS arises from its ion source. To describe briefly the process involved in ion formation the liquid containing the analyte is injected into the system from the injection syringe pump and passes through the electrospray needle, that has high potential difference in reference to the counter electrode. This results in formation of a spray of charged droplets from the needle and then the droplets are repelled from the needle towards the source sampling cone on the counter electrode and finally solvent evaporation occurs when the droplets travel between the electrospray needle and the collecting cone (Figure 8b). As the solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension provided by the solvent can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as naked charged analyte molecules. These charged analyte molecules can be singly or multiply charged and trapped to the mass analyzers. Because the formation of ions involves extensive solvent evaporation, the typical solvents used for electrospray ionization are prepared by mixing water with volatile

organic compounds like acetonitrile. The ions observed by mass spectrometry may be quasimolecular ions created by the addition of a proton (a hydrogen ion) and denoted $[M + H]^+$, or of another cation such as sodium ion, $[M + Na]^+$, or the removal of a proton, $[M - H]^-$.

The ions are then sorted according to their mass to charge ratio by the mass analyzer. In this experiment the analyzer is time of flight analyzer (TOF). In this mass analyzer the total time elapsed for a given ion to travel from the iterance point to the detector is determined by:

$$t = k\sqrt{\frac{m}{q}} \tag{7}$$

2.6 Data Treatment

The raw electrochemical data were converted into the corresponding current data matrix **I** that contains as many rows as the number of recorded voltammograms and as many columns as potentials scanned during the current measurements using a homemade programs developed in Mathlab [46]. First singular value decomposition (SVD) is applied to estimate the minimum number of mathematical components (principal components) which corresponds to the number of possible electrochemical reaction which contribute linearly to the signal. Then the matrix **I** is decomposed as the product of a matrix C (concentration of each component) and a matrix V^T (that correspond to the pure voltamograms that contribute to the current) plus an error matrix **X** [47] which is denoted by the following equation:

$$\mathbf{I} = \mathbf{C}\mathbf{V}^{\mathbf{T}} + \mathbf{X} \tag{8}$$

Where $\mathbf{V}^{\mathbf{T}}$ stands for the transposed matrix **V** and the unit of **I** and **X** is current (A), mol L^{-1} for C and A mol^{-1} L for **V**.

The above procedure is made in an itertative way from a first estimation of concentration profile or pure signals which is postulated from the data matrix. In the ALS analysis several restrictions

can be made such as selectivity (only one component exists in some part of the matrix), non-negativity (for concentration and voltammetric signals), unimodality (single peak shape of concentration profiles and or pure voltammograms), closure (mass balance application) and signal shape (fitting signals to empirical equations) [47]. The error associated with decomposition of the original current matrix is expressed as the percentage of lack of fit (lof) as the following equation:

$$\mathrm{lof} = \sqrt{\frac{\sum_{i,j}(I_{ij} - \hat{I}_{ij})^2}{\sum_{i,j} I_{ij}^2}}\, 100 \qquad (9)$$

Where I_{ij} are the element of the original current matrix I_{ij}, and $\hat{I}_{ij}$ resulting matrix calculated from the product of C and V^T by ALS analysis.

In the case that any of the signals moves along the potential axis during the titration, the linearity of the signal decreases and a correction is necessary prior to the use of MCR-ALS. This can be done by shiftfit/shifitcalc program which is developed on the Matlab. Before the correction of the potential shift estimation of the initial reference voltamograme is required by a program called peak maker on Matlab that generate Gaussian peaks according to the equation:

$$y = a \exp\left[-\frac{(x-b)^2}{c}\right] \qquad (10)$$

Whose characteristic parameters (height a, position b and width c) are selected manually with the mouse by visual comparison to the data matrix (I). The peaks are then integrated into a matrix that can be used whether as a set of reference voltammograms (V_0) to be applied in shiftfit or as an initial estimation of the pure voltammograms (V) used to start the MCR-ALS iterations. This is done from the 2D plot of the experimental data matrix by moving the curser of the mouse on the top of the possible components and pressing the mouse button. Then the parameters a, b, and c are defined and the guasian peak for each component is established on the experimental 2D

current matrix. Using the initial reference voltamograms (Vo) developed the shift correction is done. Figure 9 show that the combined application of shiftfit and shiftcalc to an experimental matrix Iexp affected by lateral movement of one or more signals.

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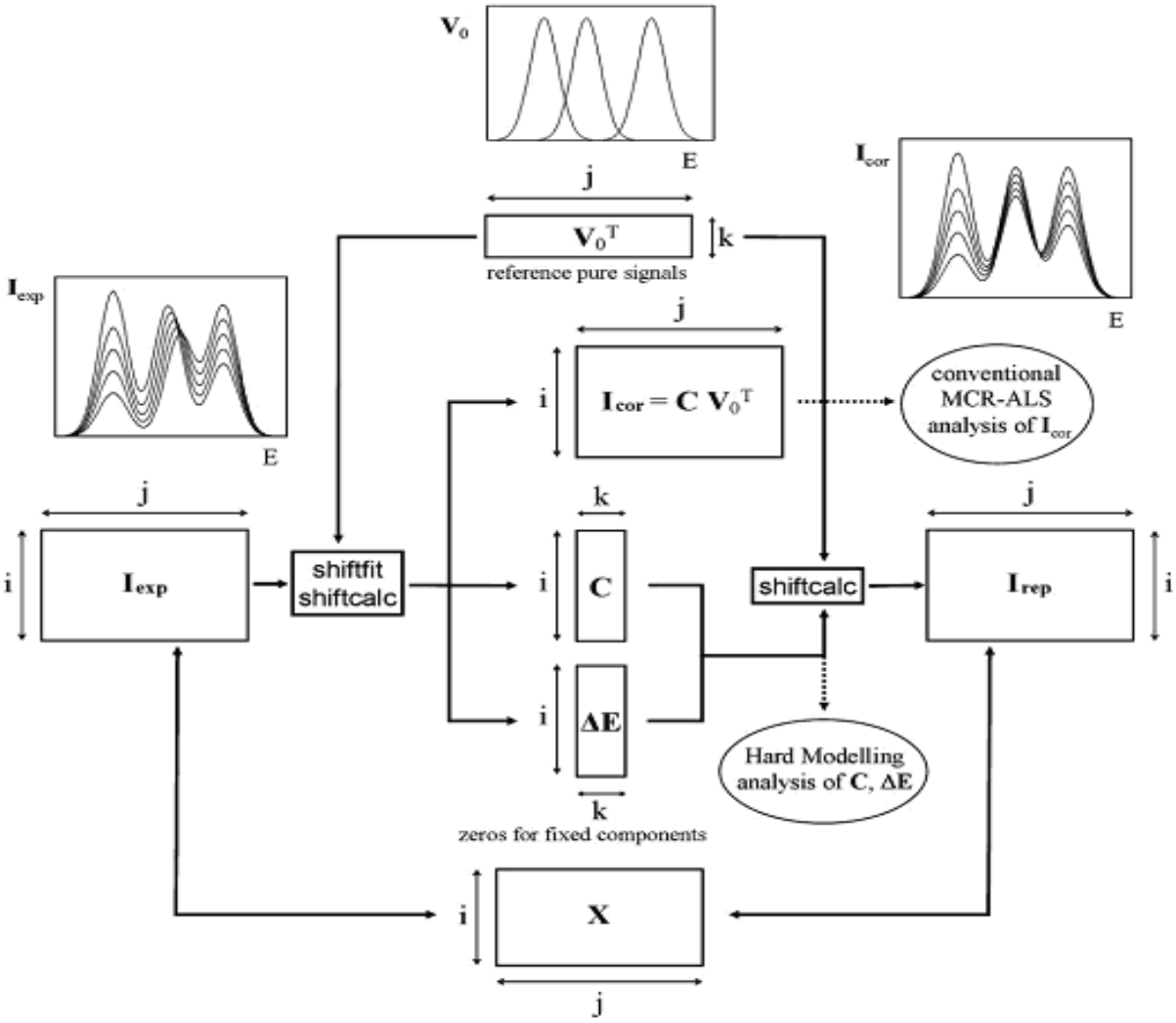

Figure9. Flowchart for potential shift correction for MCR-ALS analysis applied for experimental data matrix Iexp (A.Alberich *et al*).

To describe briefly how the function works shiftcalc displaces every voltammogram from the experimental matrix, I_{exp}, a given potential ΔE to produce a matrix I_{shift} and shiftfit optimize the value of ΔE iteratively to produce a new matrix I_{cor} that has a fixed potential as assigned in the Vo matrix (a matrix which is defined intuitively from the original experimental matrix), a concentration matrix and a potential shift matrix ΔE. Then I_{cor} can be treated by the usual MCR-ALS to obtain optimized C and V matrices and ΔE can be used in further investigation for the calculation of parameters for the complex such as stability constant by fitting hard models developed by the DeFord and Hume [48] according to the following equation:

$$F_0 = \exp\left\{ -\frac{nF}{RT}(E - E_0) - \ln\left(\frac{I}{I_0}\right) \right\} = 1 + \sum_{i\ \ 1}^{m} \beta_i (c_L)^i \qquad (11)$$

where F_0 is the Leden function of zero order, F is the Faraday unit of charge, E_0, I_0 are the characteristic potential and current, respectively, measured for the free metal ion in the absence of the ligand, E, I, are the same parameters obtained for a bulk concentration c_L of the ligand and β_i are the successive overall stability constants of the formed complexes (with i ranging from 1 to m).

RESULTS AND DISCUSSIONS

3. RESULTS AND DISCUSSION

3.1 ESI-MS Experiments

Electro spray ionization mass spectrometry (ESI-MS) is a versatile and efficient spectrometric technique that allows a definitive identification of stoichiometries of ionic complexes through interpretation of uncomplicated and easy to understand mass spectra established by a plot of relative abundance as a function of mass-to-charge ratio (m/z) for the species detected in the gas phase [23,45,50]. In electro spray mode of ionization soft energy is employed for ionization of the analyte species which enables one to get prominent peaks for unfragmented molecular ionic species in the mass spectrum unlike the other mode of ionization technique that are conventionally used in mass spectroscopic techniques. Therefore, the technique is highly desirable for the study of protein- metal complexes [50]. In this study the influence of adsorption of peptide ligands on the Bismuth film electrode on the Cd^{2+}- thiol complexation is examined to evaluate the potentiality of Bismuth-based electrodes for metal-thiol complexation studies. In addition the result of ESI-MS experiment was used to get supportive information for proposal of complexation mechanism for PC_2 and GSH with Cd under the given experimental condition.

Positive ion ESI-MS has been applied for various proportion mixtures of Cd^{2+}, Bi^{3+}, GSH and PC_2 in NH_4/AC buffer solution. In a previous investigation on metal complexation process by ESI-MS incubation of the metals-ligand mixture for 12 hour was required for total complexation reaction [50]. However in this study mixture solutions were prepared freshly to reproduce the electrochemical measurement conditions. The more intense peaks observed in the spectra are presented in Table 1 as m/z values. Peak assignments are listed according to the general formula:

$$m/z = [Cd_xBi_y(GSH)_z\text{-}nH]^+ \quad (12)$$

Where the dominant isotopic mass for Cd = 114, Bi =209, GSH= 307 and H=1 and the value of x, y, and z are selected to assign the observed m/z values. Also the value of n corresponds to the number of hydrogen that must be removed or added to optimize the observed m/z charge and mass which is equal to 2 x +3y-1.

Solution Components	Molar Ratio of the Components	Mass –to-charge m/z +ve	Assignment	Intensity (number of counts)
	2:1	179	[Cys-Gly+H]+	2.00E+05
		308	[GSH+H]+	2.00E+06
Cd,GSH		615	$[(GSH)_2+H]+$	7.90E+05
		419	[Cd(GSH)-H]+	9.00E+02
		725.44	$[Cd(GSH)_2-H]+$	4.80E+02
	1:1	179	[Cys-Gly+H]+	4.19E+05
		308	[GSH+H]+	5.00E+04
		615	$[(GSH)_2+H]+$	8.00E+05
		419	[Cd(GSH)-H]+	9.00E+03
		725.44	$[Cd(GSH)_2-H]+$	4.80E+02
	1:2	179	[Cys-Gly+H]+	5.00E+04
		308	[GSH+H]+	8.00E+05
		615	[(GSH)2+H]+	3.00E+04
		419	[Cd(GSH)-H]+	1.00E+04
		725.44	$[Cd(GSH)_2-H]+$	4.60E+03
		836.94	$[Cd_2$ (GSH)-H]+	6.20E+02
Bi,GSH	2:1	179	[Cys-Gly+H]+	4.00E+04
		308	[GSH+H]+	3.00E+05
		514	Bi(GSH)-H]+	1.00E+03
		820.9	$[Bi(GSH)_2-H]+$	3.00E+03
	1:1	179	[Cys-Gly+H]+	2.00E+05
		308	[GSH+H]+	2.00E+06
		615	$[(GSH)_2+H]+$	1.00E+03
		514	[Cd(GSH)-H]+	2.00E+03
		820.9	$[Cd(GSH)_2-H]+$	6.00E+02
	1:2	179	[Cys-Gly+H]+	8.00E+04
		308	[GSH+H]+	2.80E+05
		615	$[(GSH)_2+H]+$	4.40E+04
		514	[Bi(GSH)-H]+	1.90E+05
		820.9	$[Bi(GSH)_2-H]+$	3.00E+05

Table 1.ESI-MS for data of a binary solution of GSH/Cd(II) and GSH/Bi(III) in 1:9 acetonitrile: 20 mM ammonium acetate in water at pH 7.5.

Solution Components	Molar ratio of the Components	m/z +ve(int.%)	Assignment	Intensity (number of counts)
Cd,Bi,GSH	1:1:1	308	[GSH+H]+	1.40E+05
		179	[Cys-Gly+H]+	2.00E+04
		615.06	$[(GSH)_{2+}H]+$	4.00E+03
		514	[Bi(GSH)-2H]+	1.75E+04
		820.9	$[Bi(GSH)_2$-2H]+	3.50E+04
Cd,Bi,GSH	1:1:2	308	[GSH+H]+	1.60E+06
		179	[Cys-Gly+H]+	1.00E+04
		615.06	$[(GSH)_2$-H]+	9.50E+04
		514	[Bi(GSH)-2H]+	4.75E+04
		820.9	$[Bi(GSH)_2$-2H]+	1.60E+05
Cd,Bi,GSH	2:2:1	308	[GSH+H]+	1.00E+05
		179	[Cys-Gly+H]+	6.00E+03
		615.06	$[(GSH)_2$-H]+	6.50E+03
		514	[Bi(GSH)-2H]+	9.50E+04
		820.9	$[Bi(GSH)_2$-2H]+	5.00E+03
Cd,Bi,GSH	1:2:1	308	[GSH+H]+	7.00E+05
		179	[Cys-Gly+H]+	3.60E+04
		615.06	$[(GSH)_2$-H]+	1.50E+04
		514	[Bi(GSH)-2H]+	1.00E+04
		820.9	$[Bi(GSH)_2$-2H]+	1.60E+04
Cd,Bi,GSH	2:1:1	308	[GSH+H]+	1.60E+06
		179	[Cys-Gly+H]+	7.00E+04
		725.44	$[Cd(GSH)_2$-H]+	5.80E+03
		514	[Bi(GSH)-2H]+	1.50E+04
		820.9	$[Bi(GSH)_2$-2H]+	1.00E+05
Cd,Bi,GSH	1:1:4	308	[GSH+H]+	7.50E+05
		179	[Cys-Gly+H]+	6.00E+04
		615.06	$[(GSH)_{2+}H]+$	5.00E+05
		514	[Bi(GSH)-2H]+	7.00E+04
		820.9	$[Bi(GSH)_2$-2H]+	1.00E+05

Table 2.ESI-MS for data for ternary solution of GSH, Cd(II), and Bi (III) in 1:9 acetonitrile: 20 mM ammonium acetate in water at pH 7.5.

Characteristic natural abundance isotopic pattern of cadmium was also used for definitive assignation of the observed m/z by comparison of the experimental pattern with a statically determined pattern [50].

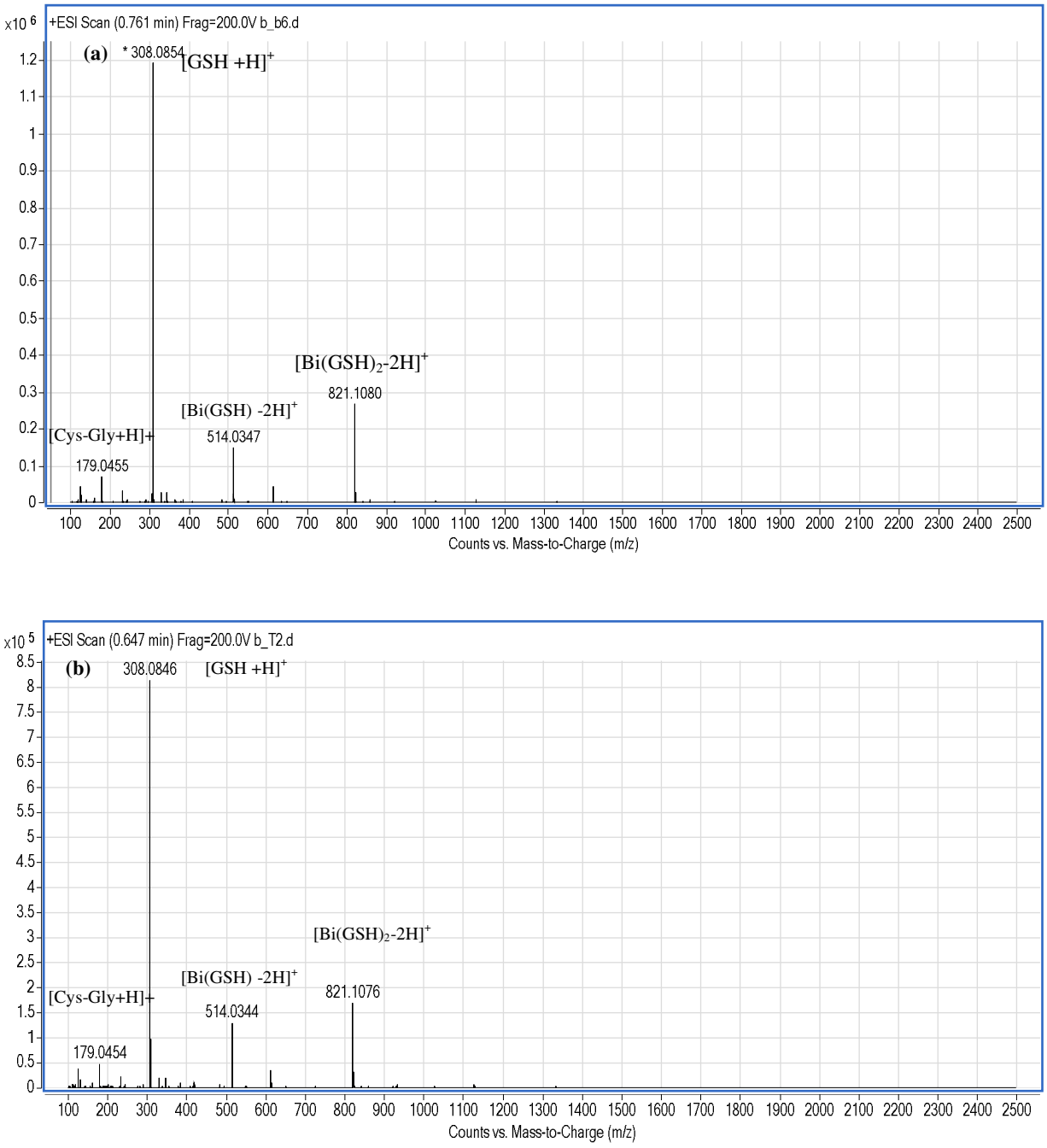

Figure10. Electrospray ionization mass spectrum (ESI-MS) for (a) 1:4 (Bi:GSH) and (b) 1:1:4 (Cd:Bi:GSH) in 10% acetonitrile-0.02 M Ammonum acetate in water.

The result of ESI-MS show that for all proportions of Bi(III), Cd(II) and GSH the dominant complex species were bismuth complex with very low intensity of some Cd-GSH complexes. For example for the mixture of 1:1:4 Cd:Bi:GSH solution the ESI-MS spectra shows dominantly $Bi(GSH)_2$ and Bi(GSH) (Figure10b) complex with minor peaks associated with Cd-GSH complexes. In addition comparison of the spectra obtained from the solution containing only Bi(III) and GSH with that containing Cd(II), Bi(III) and GSH a close similarity was observed ; therefore there is a relatively intense peak for Bi-GSH complex as compared to the Cd-GSH complex species. This observation does not lead to an absolute conclusion that bismuth complex formation is more favored than cadmium complex formation as ESI-MS may have a higher sensitivity to the former complex. However, there is clear evidence that bismuth has very high affinity to SH-bearing compounds which leads to adsorption of significant amount of GSH on to the surface of Bi-film that results in the oxidation of the electrode to form Bi-GSH complex (see the discussion part on section 3.4.3). Oxidation of Bi-film results in a release of Bi(III) to the solution which may interfere in two ways with the study of complexation of thiol-bearing peptides with analyte metal ion. First it gives rise to unwanted signal in the voltammogram which creates complexity in the interpretation of the complexetion sequence and secondly Bi(III) decreases the actual concentration of peptide which may lead to wrong prediction of stoichiometries of the complex of the main analyte metal. Fortunately the anodic signal related to oxidation of bismuth film is not very significant due to probably an inert electrochemical character of the oxidation process of Bi film to form Bi-peptide complex as compared to the corresponding mercury electrode.

It is appropriate to mention that “in situ” mode of Bi-film preparation from a solution containing Bi(III) and thiol-peptide is not a suitable technique for speciation studies of a given metal ion due to the direct interference of Bi(III) in the complexation process of the analyte metal with the thiol compounds. Therefore “ex situ” mode of film preparation seems to be the preferred technique for such study. In addition a great deal of care is required in selection of an appropriate working potential window to avoid the oxidation of Bi-film electrode even in ex situ technique.

3.2 Selection of the Working Potential Window

Previously the potential window for BiFE in stripping analysis was assessed in acetate buffer and it was pointed out that basic pH conditions give the widest potential range with minimum solvent reduction signal [33]. To confirm this fact and to find the widest potential window for the case of BiSPCE buffer solutions with varying pH values were investigated. Cyclic voltammetric measurements were done inside the potential range of -0.5 and -1.6 V for 0.01 M maleic-maleate buffer at pH 6.4 and 0.05 M borate buffer system at pH 7.0, 7.5 and 8.5. The appropriate potential window for each system is limited by reduction of solvent in the negative side and oxidation of bismuth film in the positive direction. As it can be seen from Figure 11 the widest potential window is observed in alkaline conditions (7.5 or 8.5). However there was no much difference in the potential width between pH 7.5 and 8.5 and the former is preferable for speciation studies as it is close to the natural conditions. In addition, to select the appropriate potential window considering BiSPCE +0.5 V and -1.6 V were set as first vertex and second vertex potential respectively for the system of 0.05 M borate buffer at pH 7.5. According to Figure12 the positive and negative potentials are limited at -0.25 V and -1.5 V by oxidation of bismuth film and reduction of the solvent respectively.

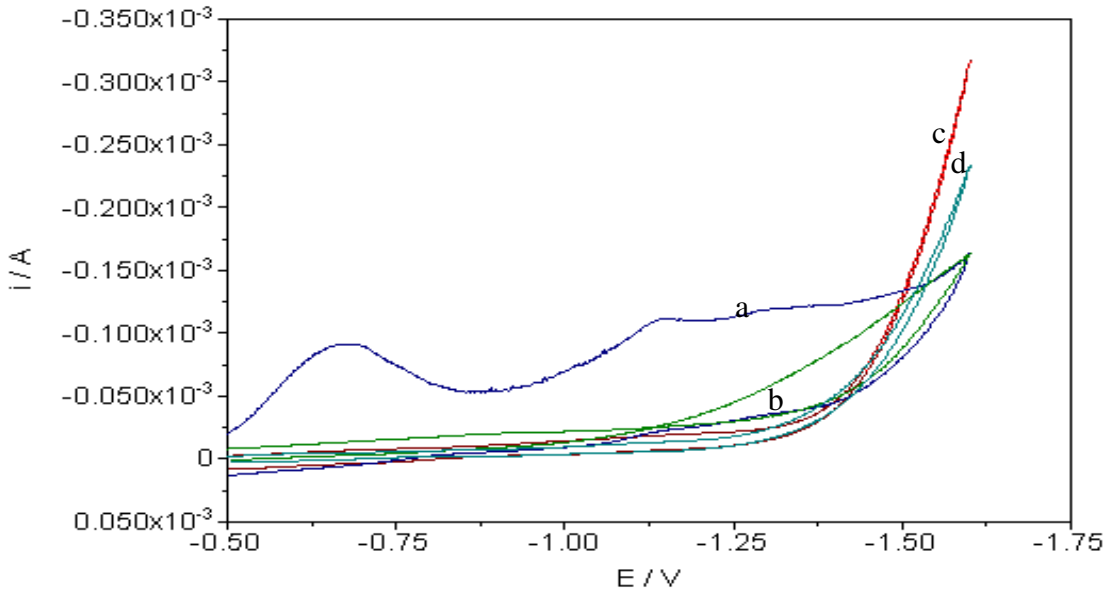

Figure11. CV Voltamograms for the different buffer solution at scan rate of 0.1V/s :(a) 0.01 maleic-malete buffer pH 6.4 (b) 0.05 borate buffer pH 7.0 (c) 0.05 borate buffer pH 7.5 (d) 0.05 borate buffer pH 8.5.

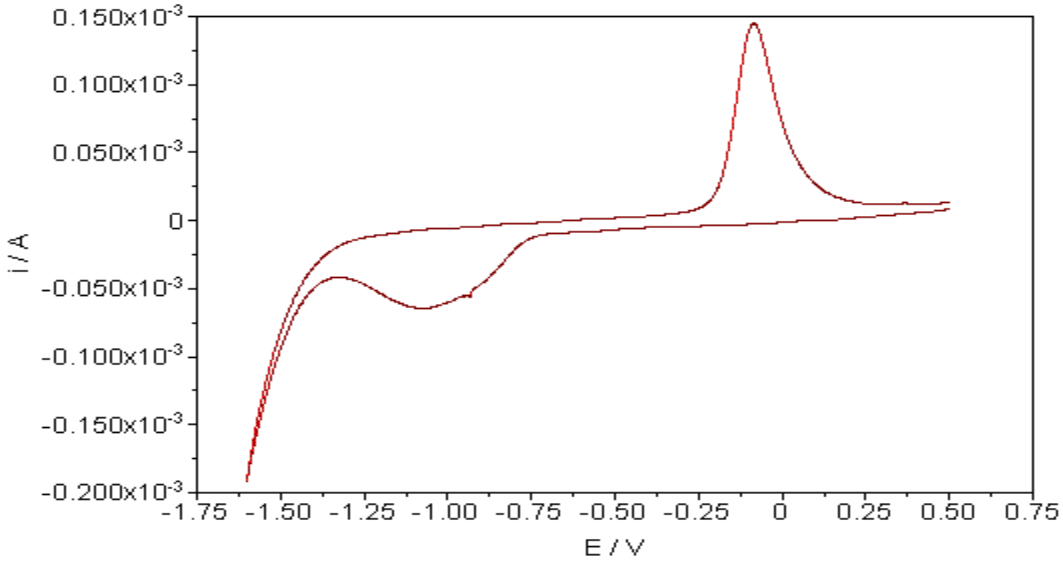

Figure12. CV voltammogram for 0.05 borate buffer solution at pH 7.5 and scan rate of 0.1V/s with first and second vertex potential at -1.6 and 0.5V vs Ag/AgCl.

3.3 Effect of pH on the Electrochemical Reaction of Cadmium Complex on BiSPCE

As a complementary work for the above preliminary investigation, the effect of pH on the electrode response for complex and free metal was investigated at various pH values. For thiol metal complexation process the removal of proton from sulfur group is required [51]. Therefore theoretically it is expected that complexation process is far to complete when the pH of the reaction medium is fairly high. Therefore it seems that higher pH value may help to improve the low sensitivity problem of bismuth based electrodes to thiol-metal complex by mounting the yield of the complex formed. However the repeatability of the peak intensity and the stability of the peak potential should be evaluated at various pH to make sure that the data obtained are suitable for further chemometrical analysis before choosing the best pH value.

The peak intensity of the complex and the free metal reduction signals were monitored at various pH values (6.4, 7.0, 7.5, and 8.0) in 0.01 M maleic-maleate buffer system at pH 6.4 and 0.05 M Borate buffer for the rest of pH values. The effect of pH on the peak intensity of 1:2 Cd: GSH is shown in Figure 13 and also the plot of peak current against the pH value can be seen in Figure 14. The peak current associated with Cd-GSH complex reduction signal increases from 6.4 to 7.5 and drops sharply for pH values higher than 7.5. In contrast, the effect of pH on the free metal

reduction signal intensity is negligible as compared to its effect on the complex signal. This suggests that the optimum working pH for thiol complexation studies on BiSPCE is at slightly basic medium. At lower pH the thiol site of the ligand molecules are prtotonated and consequently the complexation process is hindered and the signal will have very low intensity. On the other hand higher pH values possibly affect the peak intensity of the complex signal due to passivation of the electrode surface with hydroxide ions.

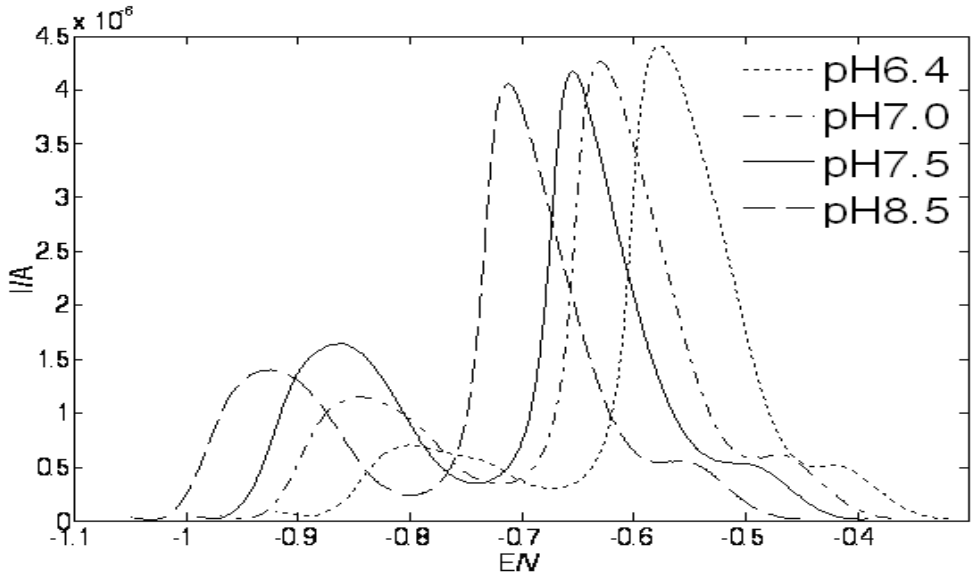

Figure 13. The effect of pH on the peak intensity of the1:2 Cd: GSH complex reduction signal ($8x10^{-5}$M GSH and Cd^{2+})

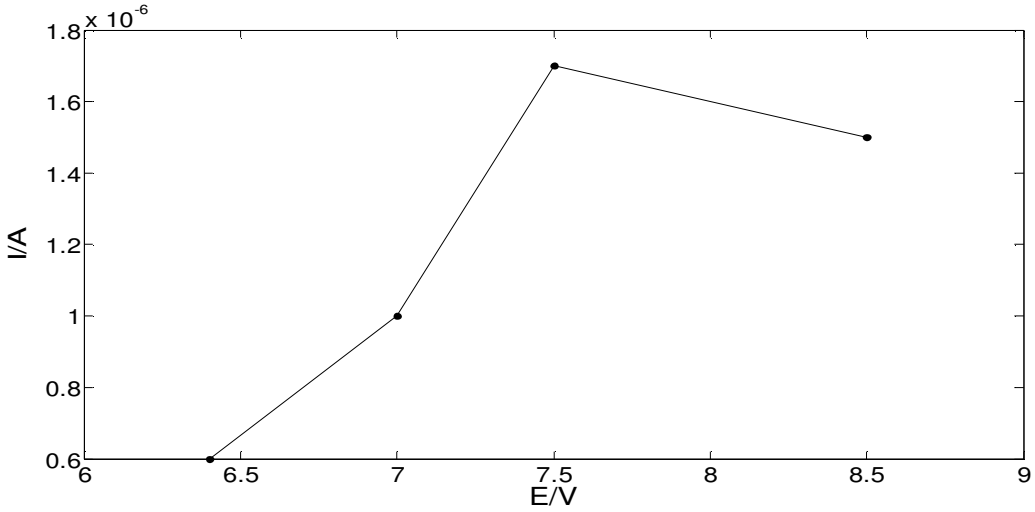

Figure14. Peak current vs pH plot showing the effect of pH on the peak intensity of the 1:2 Cd-GSH complex signal.

The stability of the reduction signal associated with the metal and the complex on BiSPCE was assessed since a highly reproducible data is required to fulfill the necessary conditions for the subsequent MCR-ALS analysis as stated previously. The peak current repeatability and peak potential stability for 1:2 GSH:Cd mixture were estimated by calculating % RSD from six repeated measurements at the various pH values. The peak intensity variability and peak potential stability for the complex signals were observed to be small at the lowest pH value (6.4) relative to the results obtained at higher pH values (Table 3). Anyways, the variability of peak intensity and stability of peak potential at all pH values were not significantly different from each other. Similar observations were seen for the free metal reduction signal. However significant potential shift was observed for both free metal and complex signals going from lower pH values to the higher values as potential is dependent on the pH of the medium [51]. Generally for reversible system the peak potential (half-wave potential) and pH of the medium at 25^{o}C are related as:

$$E=Econt-(mRT/nF)pH \quad (13)$$

Where m and n corresponds to the number of protons and electrons involved in the electrode reactions, respectively [51]. In consistency with this fact the peak potentials for the reduction of the complex signal were observed on average at -0.80, -0.85, -0.88, and -0.940 at pH 6.4, 7.0, 7.5 and 8.5 respectively.

From the potential window optimization and the study of the effect of pH on the current and peak potential it is reasonable to select pH 7.5 for further complexation study of cadmium with thiol-peptides in borate buffer on BiSPCE.

pH	Average peak current, Ip(A)	Average peak potential, Ep(V)	CV% of Ip	CV% of Ep
6.4	$6.00x10^{-7}$	-0.80	3.5	0.58
7.0	$1.05x10^{-6}$	-0.85	5.3	0.56
7.5	$1.75x10^{-6}$	-0.88	7.9	0.61
8.5	$1.50x10^{-6}$	-0.94	11.2	0.63

Table3. Repeatability of Peak current and stability of peak potential (n= 6) at various pH values.

3.4. Free Metal Reduction Signal on the Absence of Ligands

3.4.1 Free Metal Reduction Signal on the BiFE

To figure out thiol metal complexation process it is worth to see the electrochemical behavior of the free metal and free ligand on BiSPCE and BiFE at the current experimental condition. In a recent paper it was pointed out that the reduction signal of Cd(II) splits on BiFE in both differential pulse stripping voltammetric and stripping chronpotentiometric analysis [27]. For this reason the concentration range of Cd(II) that give reduction signal profile on the BiFE similar to the signal observed on MFE was also investigated to apply BiFE for complexation studies in the same manner as the corresponding MFE [34]. The signal splitting of cadmium reduction on BiFE was also observed in this study in borate buffer solution at pH 7.5. In the beginning two peaks were observed one at -0.7 and the other at -0.85V (Figure 15). The peak at the more positive potential stabilizes at higher cadmium concentration while the other peak increases up to the cadmium concentration of around $5.5x10^{-5}$ M and then it starts to be splitted to give a new third peak (Figure 15). The old peak is stabilized while the new third peak increases continuously with increasing cadmium concentration.

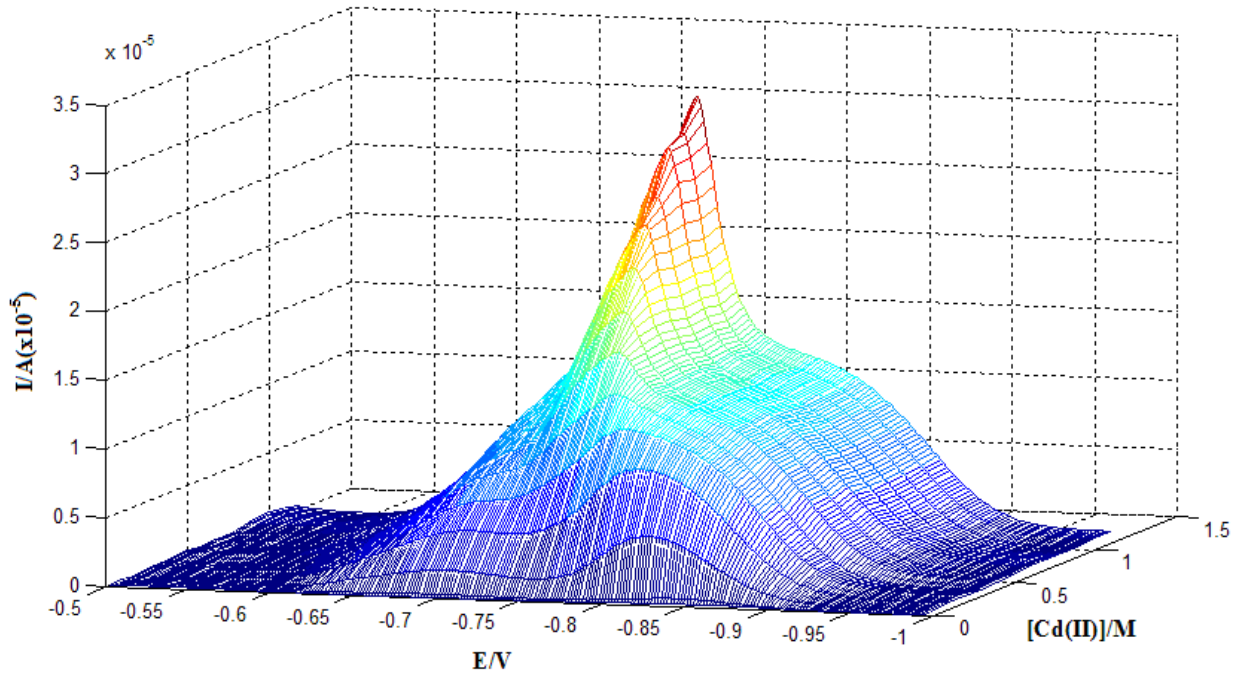

Figure15. Differential pulse voltammogram of Cd(II) on BiFE at different concentration of Cadmium in Borate buffer at pH 7.5

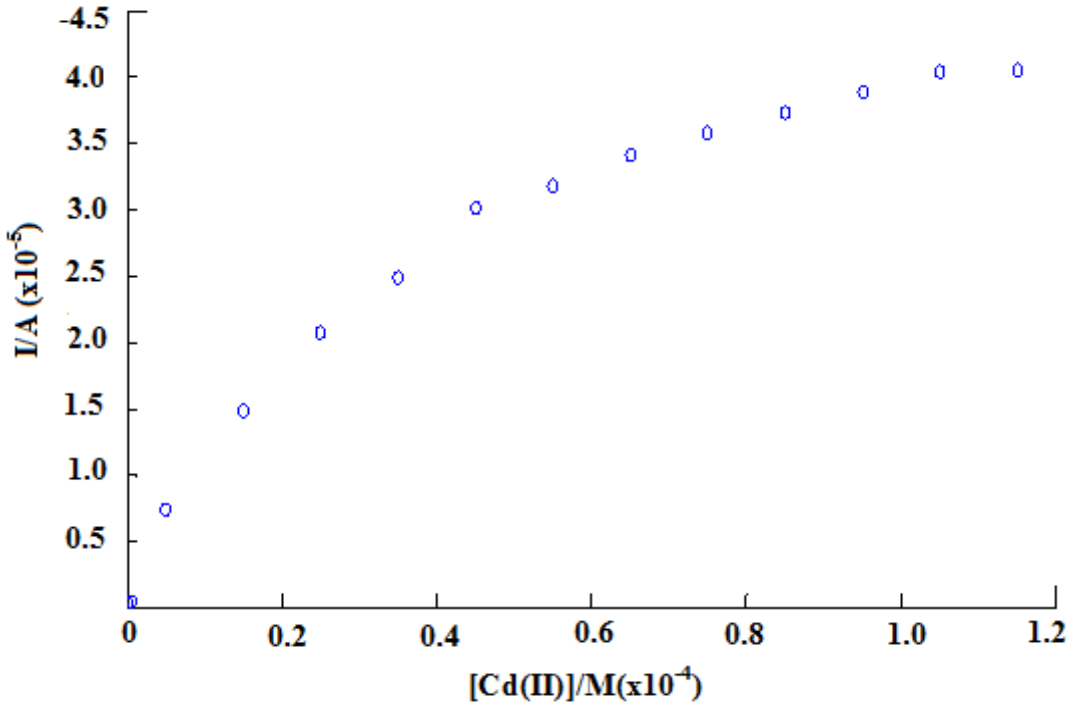

Figure16. The corresponding calibration curve for Cd(II) in borate buffer at pH 7.5

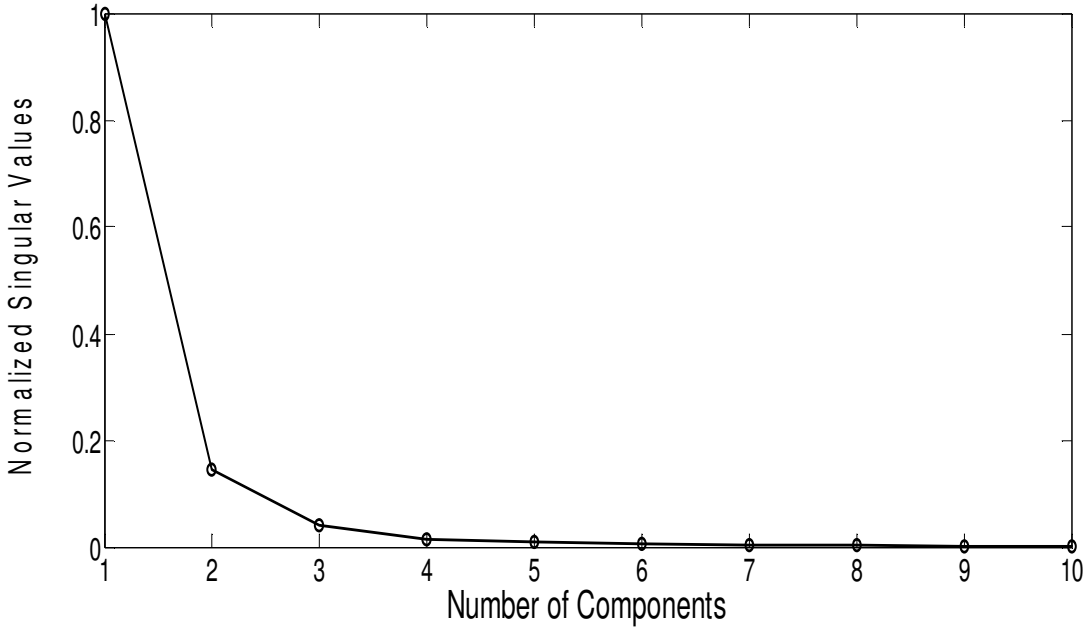

Figure17. Normalized singular value generated from the data matrix shown in Figure 15.

Even if only a single species, free metal, exist in the system MCR-ALS analysis was applied to the experimental data matrix for a better understanding of the signal splitting pattern. Starting from the result of SVD (Figure17) for the data matrix (Figure15) three components system was assumed with constraints of non-negativity for both signals and concentrations and signal shape for all components. However the lack of fit was very high (27%) and this is mainly because of the continuous movement of the potential of the peaks under this experimental condition which decreases the linearity of the data. Anyways, there is a clear evidence for the presence of Cd(II) reduction signal splitting phenomena on BiFE in borate buffer at pH 7.5. In spite of the splitting behavior of the signal of free Cd(II) quantitative analysis of the free cadmium metal is still possible as the total area of all peak should be directly proportional to the concentration of Cd(II) present in the solution. Figure16 shows the calibration plot of peak area against the concentration of Cd(II) in the solution. A deviation from linearity was observed for higher cadmium concentration due to the BiFE's surface saturation.

3.4.2 Free Metal Reduction Signal on BiSPCE

In contrast to BiFE a notable signal splitting was not observed in the reduction signal of Cd(II) at pH 7.5 (borate buffer) when BiSPCE electrode was used (Figure 18). This may be a good improvement of bismuth film electrode as it minimizes the complexity that arises from signal splitting when the thiol– metal complexation sequence is studied on this electrode. Specifically this property of BiSPCE is essential for evaluating stoichiometries and stability constants of metal complexes with various ligands.

The comparison between BiSPCE and BiFE reveals that the sensitivity for Cd(II) reduction is higher on BiSPCE electrode (Figure 19), which could be due to the higher electrode area of the screen printed electrode and rough surface structure (Figure20) which provides a better condition for platting of bismuth that consequently enhance the reduction of Cd(II) from the bulk solution. In addition the corresponding linearity range was wider on BiSPCE in comparison to BiFE which ensures the potentiality of BiSPCE for metal determination and titration in a relatively wide concentration range.

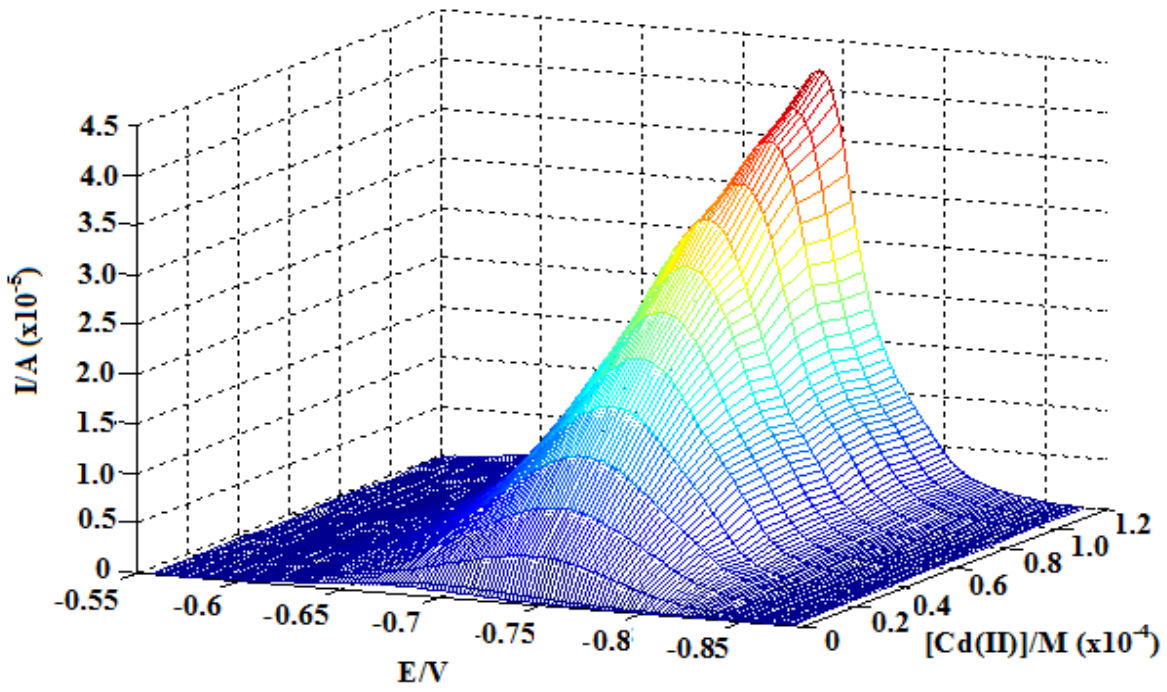

Figure 18 Differential pulse voltammogram on BiSPCE for increasing addition of Cd to borate buffer at pH 7.5.

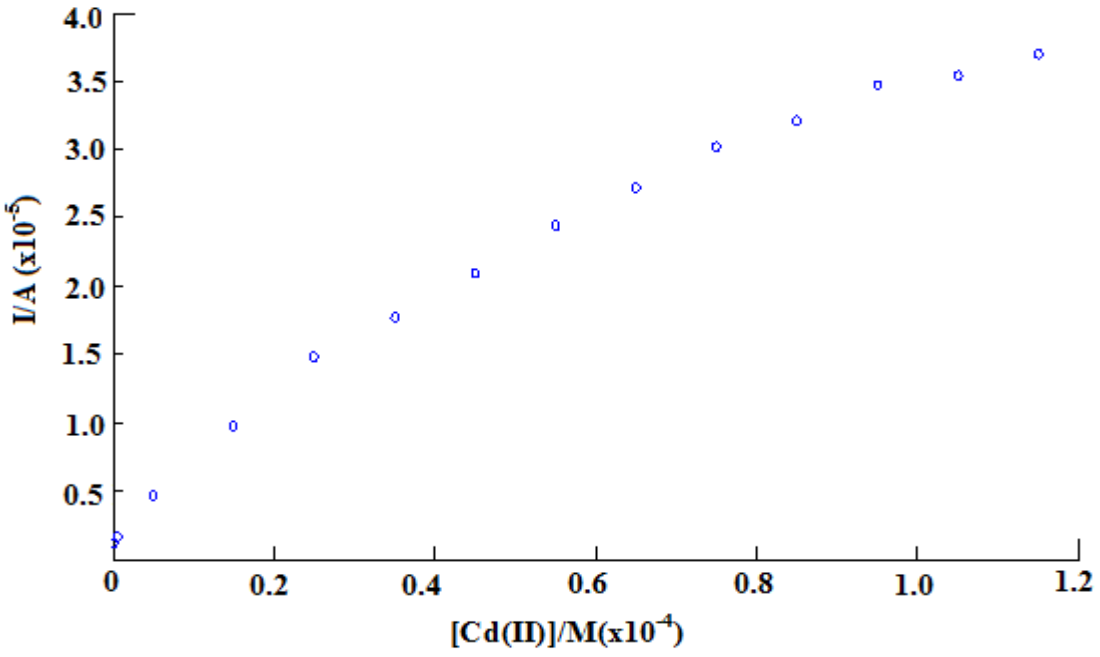

Figure19. The corresponding calibration curve for Cd(II) in borate buffer at 7.5

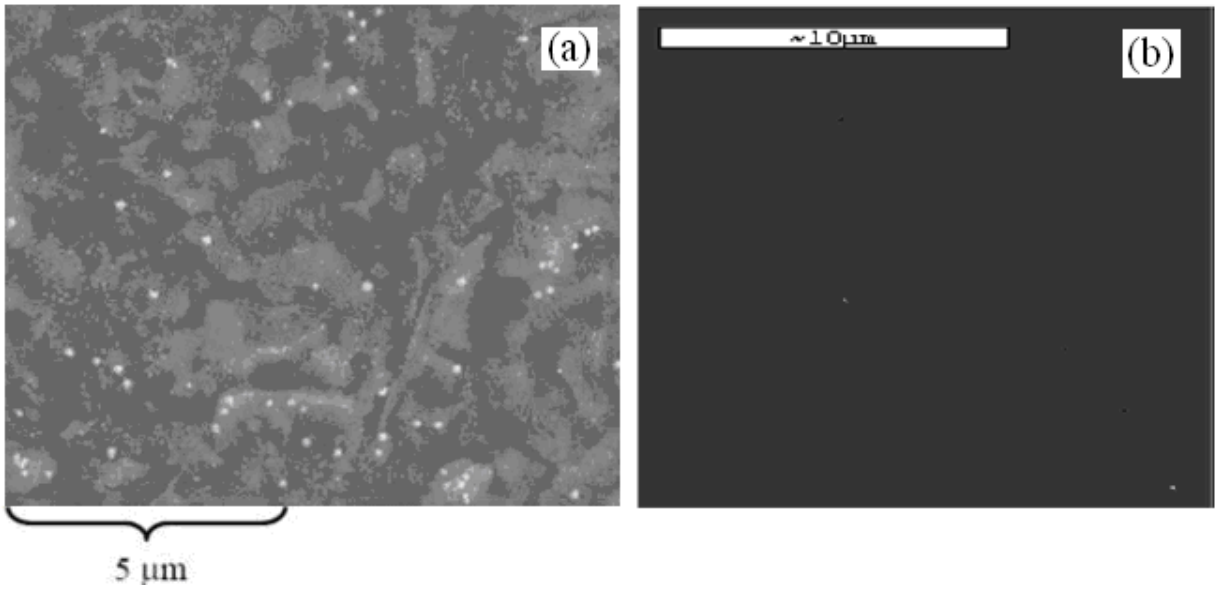

Figure20. Scanning electron microscopy images of: (a) a bare screen printed carbon electrode; (b) a bar glassy carbon electrode (A.Economu, *Trends in analytical chemistry* 24 (2005) 334).

3.4.3 Free Ligand Reduction Signal on SPCE and BiFE

As observed for the case of mercury electrodes [52] it is believed that thiol compounds are adsorbed on the other type of electrode material including bismuth film electrodes according to the following:

$$nGSH + Bi(0) \longrightarrow Bi(GSH)_n(ads) + nH + ne^- \quad (14)$$

Hence, the diffusion controlled oxidation signal that corresponds to the above chemical reaction corresponds to the oxidation of bismuth followed by the formation of bismuth–thiols adduct (Equation 14). The anodic current does not diminish to the charging current, as would have been expected if bismuth surface had been covered with the thiol blocking layer. This confirms that the thiol species continuously react with the bismuth film surface as long as the potential is fixed more than - 0.63 (see the following discussion).

In a previous study of lead-phytochelatin complexation on BiFE in maleic-maleate buffer (pH 6.4) anodic oxidation of the electrode material by the free thiol peptides was observed as a broad

peak in the region between -0.6 to -0.8 V vs Ag/AgCl reference electrode where the free lead and its complex reduction signal were also observed [35]. In consistency with this finding the anodic oxidation of the electrode material in the presence of peptides was also observed at pH 7.5 on the BiFE and BiSPCE. Two peaks at around -0.49 and -0.63 V were observed for $1x10^{-4}$ M GSH solution in borate buffer at pH value of 7.5 as shown in Figure 21. In a similar way as for the free Cd(II) reduction study, voltammogram were recorded for increasing concentration of GSH using both electrodes but the relationship observed between the peak current and the concentration of the ligand was somewhat undefined.

It should be noted that the peak current recorded for concentrated GSH ($1x10^{-4}$ M) was relatively very low (Figure19) which shows that anodic oxidation of the electrode material in the presence of GSH was also very low. The weakening or the absence of the anodic signal of bismuth electrode in the presence of thiol-peptide is an important advantage over the corresponding mercury film electrodes as this simplifies the interpretation of the signal in order to understand the complexation process.

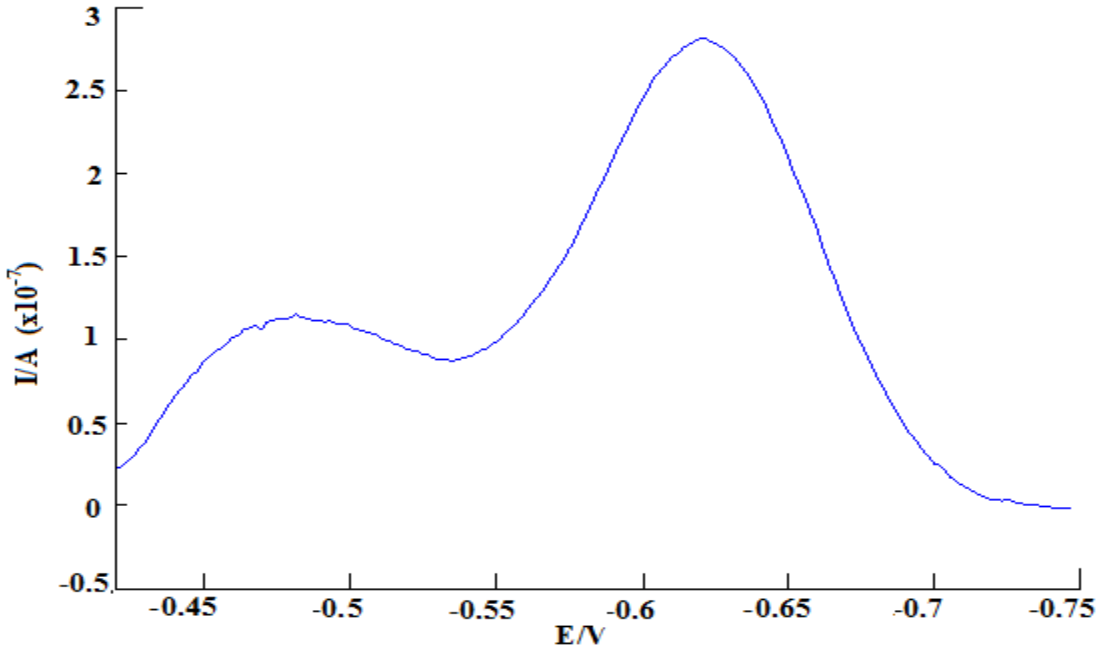

Figure21. The voltamogram of $1x10^{-4}$ M free GSH M in 0.05 borate buffer solution (pH7.5)

3.4 Complexation Study of Cd with Glutathione

3.4.1 Adsorptive Cathodic Stripping Voltammetry

Study of thiol complexation at lower metal concentration is advantageous to model exactly the detoxification mechanism at cellular level. In addition at lower concentrations of the peptides and metals the signal intensity and concentration of all the chemical species show linear relationship which allows applying MCR-ALS chemometrical methodologies to extract abundant information for the complexation sequence. An attempt was made to study at lower concentration (starting from $1x10^{-6}$M) however no signal was observed for the complex species except an intense signal associated with the free metal reduction signal.

Consequently, adsorptive cathodic stripping voltammetry was applied to accumulate complex species on to the the Bi-film electrode surface by applying a constant potential for a given period of time and then latter scanning in the negative potential direction to reduce the accumulated species quantitatively. To select the best value of the key experimental parameters (i.e. the adsorption potential and the accumulation time) various adsorptive cathodic stripping voltammetry experiments were done. First the optimum adsorption potential was searched by applying potential of -0.5, -0.6 and -0.7 V vs Ag/AgCl reference electrode and then latter scanning from -0.4 to -1.1V. It was observed at -0.6V a better signal enhancement. Next considering this potential the optimum accumulation time was selected from 60, 90,120,180 and 240s and the signal intensity was not observed to improve after 120s consequently this accumulation time was taken as the optimal time.

Therefore, -0.6 V and 120 s were used as the adsorptive potential and accumulation potential respectively for the study of Cd-GSH complexation by adsorptive cathodic stripping analysis at concentration of $1x10^{-6}$ M for both Cd^{2+} and peptide. However a well established pattern between the signal intensity of the complex and concentration of the metal and the petide could not be seen. This is partly due to none reversible electrochemical character of these species as a consequence of their strong adsorption on to the surface of the electrode. Therefore differential

pulse voltammetric technique was applied at higher concentration of the metal and the ligand ($2x10^{-5}$ M) for qualitative purpose of interpretation.

3.4.2 Cd-GSH System on BiFE

To see the evolution of the complexation process differential pulse voltammetric titrations were done in two ways: addition of $1x10^{-3}$ M Cd to $2x10^{-5}$ M GSH solution and the reverse process, addition of $1x10^{-3}$ M GSH solution to $2x10^{-5}$ Cd solution in borate buffer solution at pH 7.5. However, the first mode of titration gave more detail and sufficient information and was selected to explain the complexation process. In this mode of titration, before the first addition of Cd to the GSH, a small peak at around -0.63 V was observed which further decreases and disappears after addition of cadmium solution as shown in Figure 22. By analogy to the previous study of SH-bearing compounds complexation with metals on BiFE [34] and to the signals observed for free GSH on BiSPCE in this study; this signal is associated with the anodic oxidation of Bi electrode in the presence of GHS. By comparing the peak potential of the free Cd(II) in the absence of ligand with that of the peak located at -0.85 V it can be concluded that this reduction signal can be related to a fraction of free Cd(II) associated with an electrochemically labile complex as its potential position continuously shifts to the more positive side with increasing concentration of Cd(II). Also the peak observed at -0.95 V is related to the reduction signal of 1:2 Cd:GSH complex according to A.Alberich *et al* [34] which reports stabilization of a similar signal at Cd:GSH ratio around 0.5. In this study the anodic signal associated with Cd-GSH complex was not very important and could not be clearly seen in the expected potential region (by the positive potential side close to the free metal reduction signal). However its existence is clear, since it was observed in the beginning of the titration and before it was dominated by the intense signal of the Cd(II) reduction. In the Pb-GSH complexation study on BiFE the anodic signal was observed overlapped with the free metal reduction signal. Howevere in the case of Cd-GSH complexation, the anodic signal was well separated from the Cd(II) reduction signal.

MCR-ALS was not applied for this case as the free metal concentrations are out of the linear range and due to the presence of anodic oxidation signals of the free peptide under this

experimental condition whose evolution pattern is quite unpredictable. In addition the shape of the free metal reduction voltammograms changes continuously which hinder the correct application of MCR- ALS methodology. However the observed signals are relatively well separated as compared to the corresponding signals observed on mercury electrodes [51] therefore there is a way to analyse the trend of the pure components during the titration process qualitatively. To do so the peak intensity of free metal and the complex signals were plotted against the ratio of cadmium added to the initial peptide concentration (Cd:GSH) (Figure 23).

Figure 23a shows that as the Cd:GSH ratio increases, the 1:2 Cd:GSH complex reduction signal (located at -0.94 V) grows sharply to reach a plateau at a concentration ratio of 0.5. This result is quite consistent with a previous study made using polarographic techniques [51]. Figure 23b shows the evolution of the Cd(II) reduction signal which includes the labile complex and the free metal. The attempt to explain the evolution of the free metal and the labile complex concentration profile was not consistent with a previous finding as the free metal is not expected to exist before the ratio of 0.25 [51]. Also a change in the slope is expected at Cd:GSH ratio of around 1.0 due to the appearance of free metal but this was not observed in this case as Figure23 b shows. The most probable reason for this deviation is the splitting of the free metal signal which creates ambiguity for the assignment of the peaks for the free metal and labile complex. In the case of Pb-GSH complexation study on BiFE where signal splitting of free metal reduction signal is absent a clear change of peak intensity and peak potential shift was observed which means the appearance of free lead at higher Pb:GSH concentration ratio [34]. Therefore to get clear information about the complexation process of Cd-GSH system either the concentration of the metal ion should be very low, in the region where signal splitting is absent, or a different electrode should be used. Then, the next step is to try if BiSPCE can improve the drawbacks of BiFE, especially taking into account the absence of peak splitting for the free metal alone.

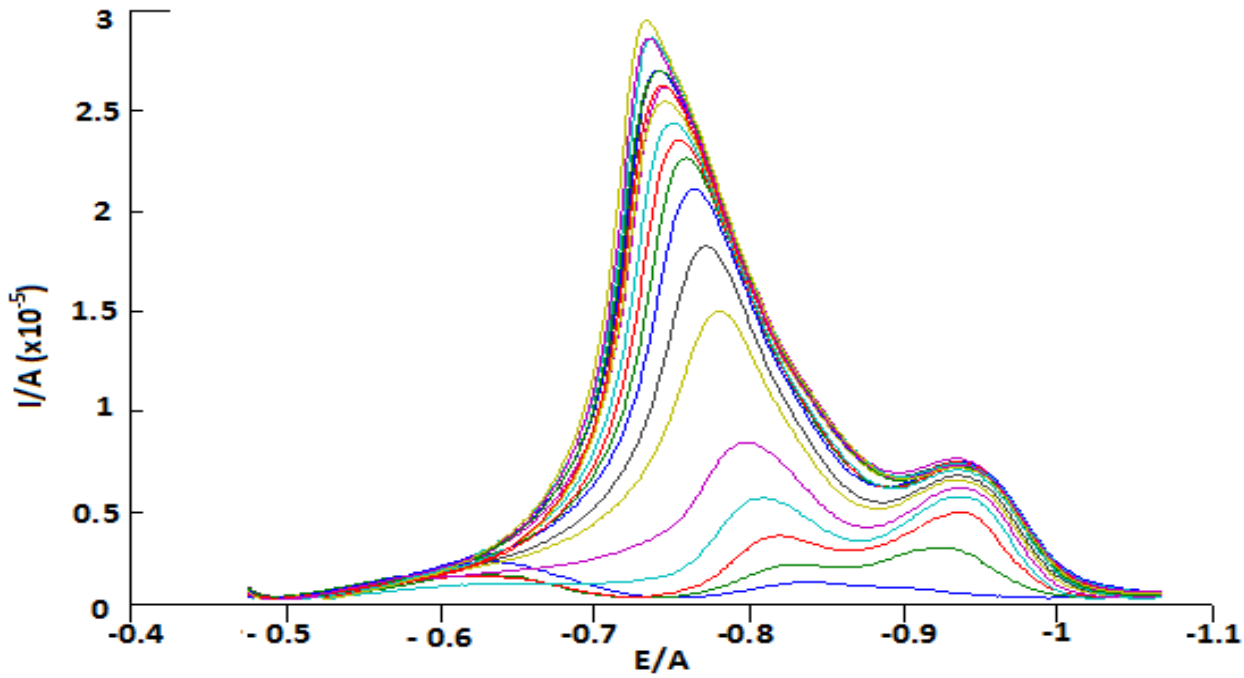

Figure 22. Differential voltamogram s measured during the titration of $2x10^{-5}$ M GSH with Cd solution in borate buffer at pH 7.5 by using BiFE.

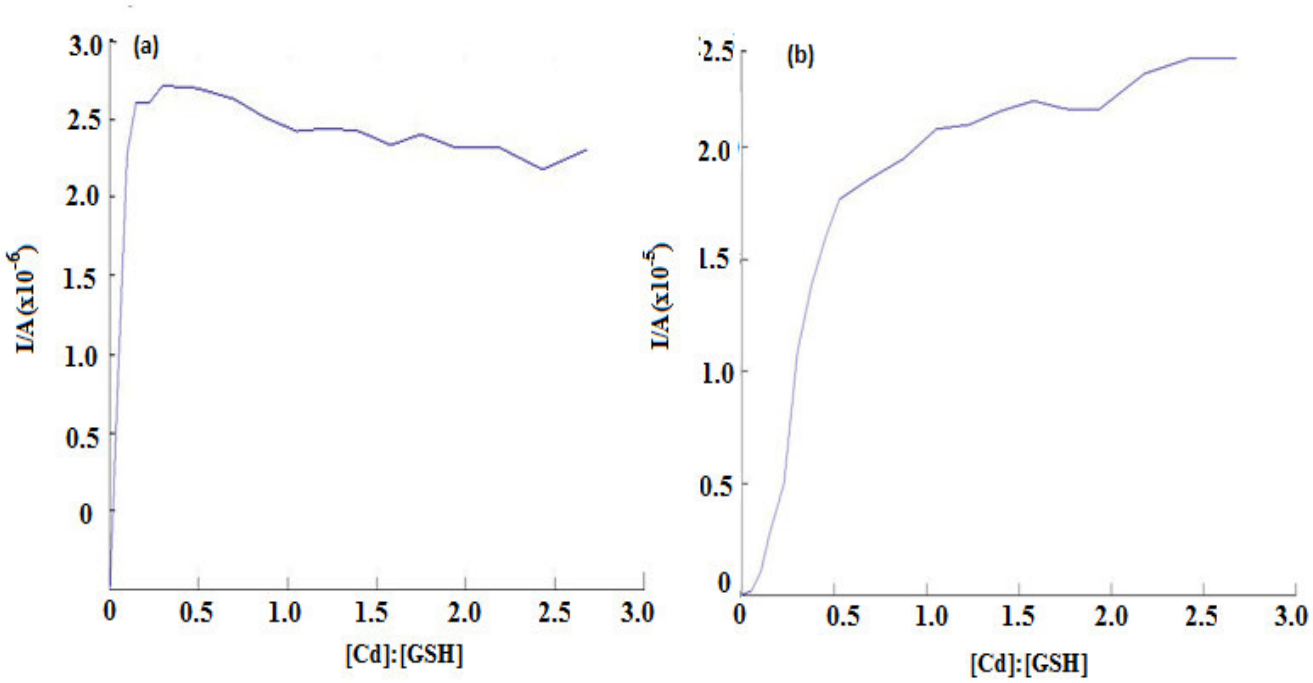

Figure 23. Peak current vs [Cd]:[GSH] ratio in borate buffer solution (a) for the complex signal and (b) for the labile and free Cd reduction signal.

3.4.3 Cd-GSH system on BiSPCE

As stated above a better information is expected from the use of screen printed electrodes since the signal splitting of the Cd(II) reduction is absent . An improvement in the peak intensity of the signals is also expected as observed from the comparison of BiFE and BiSPCE sensitivity for the metal. Comparing the results obtained using both electrodes, it can be seen that all the signal that appear on BiFE are also present in BiSPCE (Figure 24). However the metal reduction signal on BiSPCE is sharper than the corresponding in BiFE and the peak potentials for the free metal and labile complex are relatively stable (the lateral movement along the potential axis is reduced). The difference in shape of the voltamograms is associated with the absence of splitting of Cd(II) reduction signal on BiSPCE. As in the previous case, MCR-ALS analysis could not be applied for BiSPCE due to the loss of linearity of the free metal reduction and the non familiar shape of the voltamograms. However, as Figure 24 shows all the voltammograms are well separated and qualitative analysis can be used for explanation of complexation process.

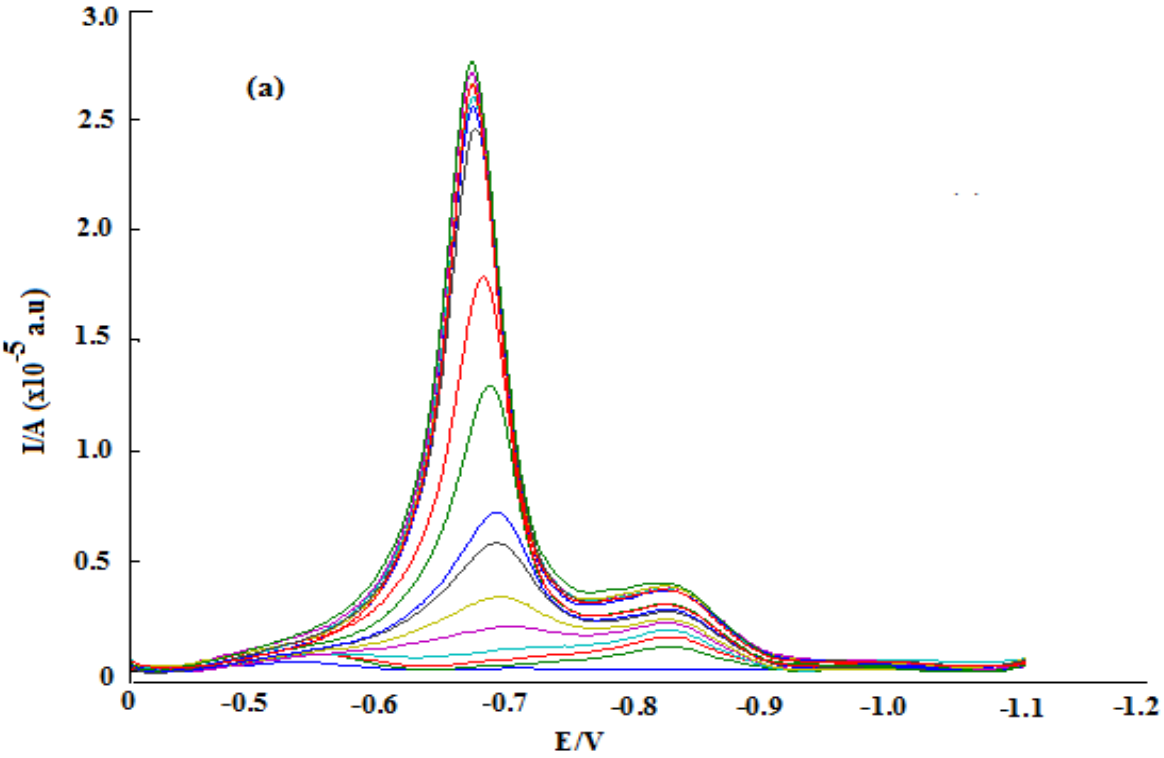

Figure 24. Differential voltamograms measured using the titration of 2x10^{-5} M GSH with Cd solution in borate buffer at pH 7.5 by using BiSPCE.

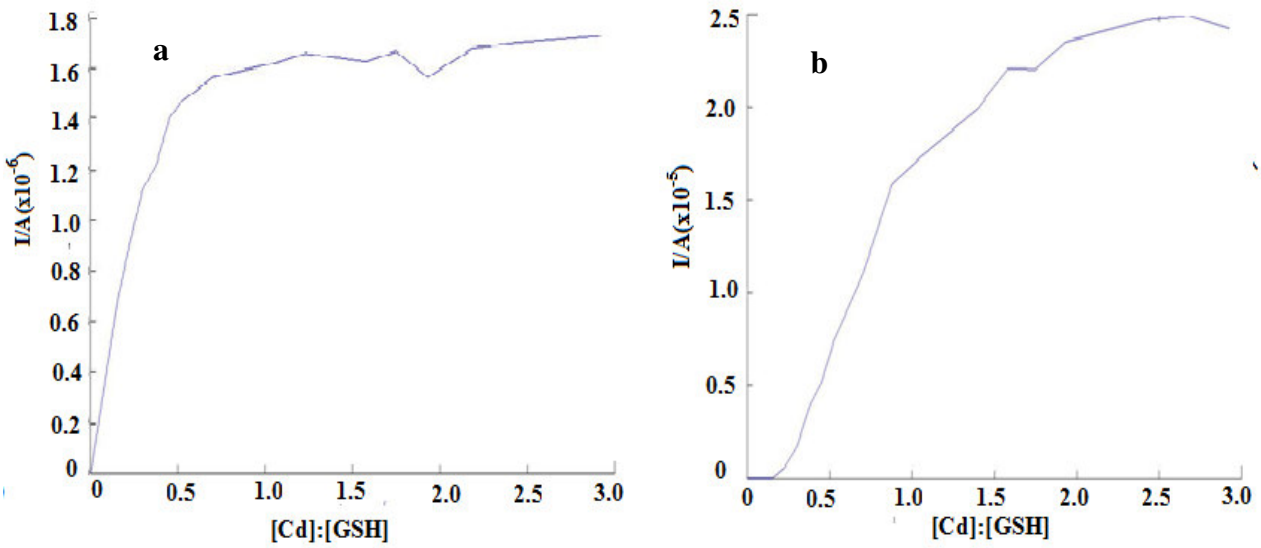

Figure 25. Peak current intensity vs Cd:GSH rato (a) for the 1:2 Cd:GSH complex and (b) for the total labile and free metal cadmium reduction signal

However it should be stated that some voltamograms recorded by this electrode had very different peak potential position which was not observed in the case of BiFE. This indicates that BiSPCE has lower reproducibility as compared to BiFE. Consequently these voltamograms were removed from the data matrix before the data treatment step.

Like in the case of BiFE, at the beginning of the titration only the anodic signal for the free GSH is observed , a signal which decreases with further addition of Cd(II) solution as shown in Figure 24. The peak current associated with the 1:2 Cd:GSH reduction signal increases sharply and levels off when 0.5 Cd:GSH concentration ratio is reached (Figure 25a) as observed in BiFE and other previous studies [34,51]. The peak potential for this complex is quite stable, (Figure 24) which is quite consistent with previous observations showing the inert nature of Cd-GSH formed between two GHS molecules and one Cd ion through the sulphur group of the ligand (Figure 26).

With regards to the Cd(II) reduction process from the labile complex and free metal , Figure 25b shows the total Cd(II) reduction profile contributed from the labile complex and the free Cd(II). Accordingly, until a concentration ratio of 0.25 reduction of Cd(II) contributed from the labile complex was not seen and this tells us that in the beginning of the titration Cd(II) ion forms

preferably 1:2 stable complex and from the ratio of 0.25 reduction of Cd(II) starts and increases sharply until the concentration ratio value of 1.0 is reached. Previously it was suggested by M.S.Diaz-Cruz,*et.al* [51] that in this concentration ratio region the incoming Cd(II) is expected to form complex of 2:2 Cd:GSH where the incoming Cd(II) is complexed probably with carboxylate part of gluthation which was already complexed in 1:2 Cd-GSH with sulphur group as shown in Figure 27.

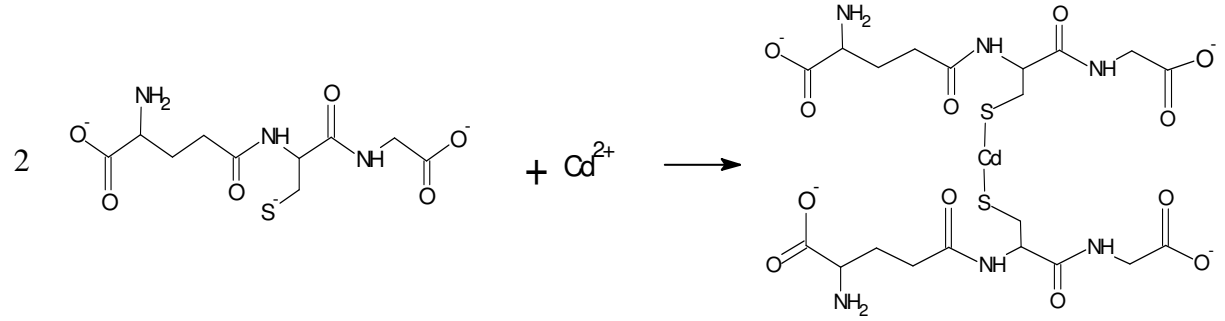

Figure 26. Cd-GHS complex formation reaction at low Cd:GSH ratio

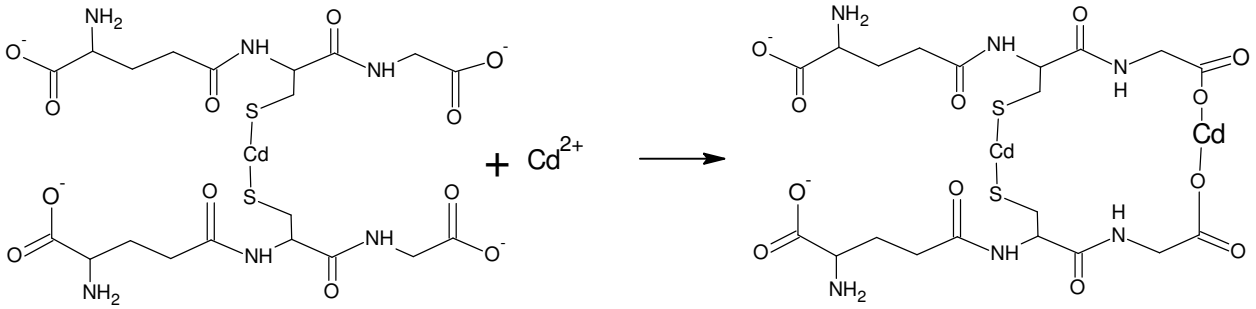

Figure27. Complex formation reaction for higher Cd:GSH ratios

Therefore in the concentration ratio region above 0.25 the labile and stable complexes exist simultaneously even at higher metal concentration. This signifies that the electrochemical process of the first complex (the stable one) is not substituted by the electrochemical response of the second complex and the two complexes interact as shown in Figure 27. However, at higher concentration ratios the dominant species is expected to be the 2:2 Cd:GSH complex. For higher concentration ratio (Cd:GSH > 1) the free metal reduction signal starts to appear which is clearly observed from the bending of the curve (Figure 25 b). The deflation of this curve is a result of the loss of sensitivity of the electrode when its surface gets saturated by excessive metal ion present in the solution.

From the above observation, it can be concluded that BiSPCE can be applied for speciation studies of metals in the presence of relatively simple thiol peptides like GSH. Particularly for metals like Cd(II) and Zn(II) whose reduction signal split on BiFE the use of BiSPCE is the best optionto get comparable result with that of the conventional mercury electrodes whose use is becoming unacceptable due to the adverse environmental implications.

3.5 (γ-Glu-Cys)$_2$Gly Complexation with Cadmium Study on BiSPCE

Toevaluate the applicability of BiSPCE for metal complexation studies with phtochelatins PC_2-Cd complexation sequence was studied and the result was compared with previous studies. In a similar way as in the study of Cd-GSH complexation, direct titration (i.e addition of 10^{-3} M PC_2 into $2x10^{-5}$ M Cd^{2+} in 20 ml of buffer solution) and indirect titration (addition of $1x10^{-3}$ M Cd^{2+} to $2x10^{-5}$ M PC_2) were performed. As the latter titration mode gave sufficient information therefore it was selected to discuss the complexation process taking place between Cd and PC_2. Figure 28b shows the mesh plot for the current matrix produced during the titration process. Before the addition of Cd^{2+}, anodic oxidation of the bismuth electrode (signal 1) was observed at -0.65V which decreases and disappear upon the addition of Cd^{2+} solution. Again as the case of Cd-GSH complexation anodic oxidation of the electrode material due to the presence of Cd- PC_2 complex was very weak as compared to the case of mercury electrode [25]. Further additions of Cd^{2+} produce a signal associated with complex reduction (signal 4) at around -1.0 V and then a signal appears at -0.75 V (signal 3) . Finally the free metal reduction signal is observed at high metal to thiol ratios (signal 2).

To understand the concentration profile of the individual species present in the system during the titration (free metal, complexes and free ligand) the usual MCR-ALS analysis was applied with four component: one for the metal ion, two for the successive complexes and one for the free ligand with the constraints of non-negativity and signal shape. But the lack of fit was much more than the acceptable limit (27%) and for this reason the number of components was varied to lower the error. However no improvement was observed. This is mainly due to loss of linearity by the lateral movements of signals of some components along the potential axis [48].

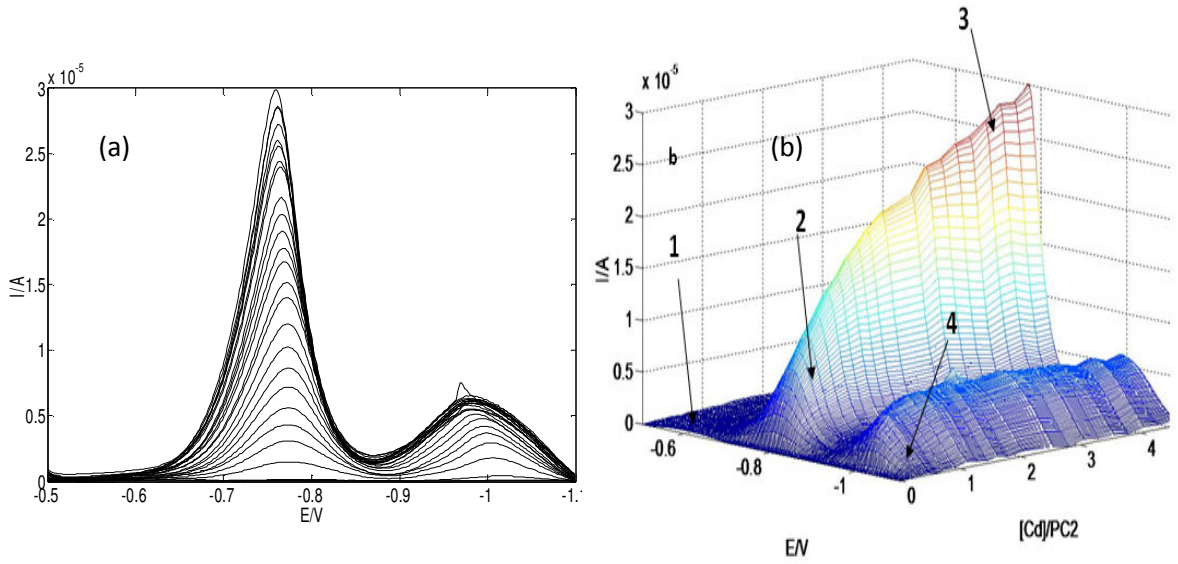

Figure28. (a) The voltammogram profile of the titration of $2x10^{-5}$ M of PC_2 with $1x10^{-3}$ M of Cd^{2+} in borate buffer solution at pH 7.5 (b) the corresponding mesh plot of the current data matrix.

Due to this reason the alternative method was used, i.e. the shift of the potentials for those signal whose peak potential is not at fixed position were corrected by a program shifcalc/shiftfit before resolving the full data matrix by MCR-ALS as explained in section 2.6. Then the concentration profile (C matrix) is obtained for all components and also additional information, shift potential (ΔE), is obtained which can be used for estimation of the stability constant of the labile complex. In our case the best result was obtained by considering four components where three of them have moving peaks along the potential axis while the remaining one shows stable peak potential. A visual inspection of the experimental matrix depicted in Figure 29 a shows that all the signals were moving except the first one (anodic oxidation of the electrode material associated with the free peptide). Due to the weak intensity of this anodic signal, it can hardly affect the linearity of the data even if there is a significant movement along the potential axis. Therefore potential shift correction was not applied for this signal ($\Delta E = 0$).

After peak potential movement correction by shiftfit/shiftcal with reference to the unitary voltamogram, Vo (Figure 29 a) MCR-ALS was applied by assuming the four components defined by a single peak and applying the constraints of non-negativity (for both concentrations

and signals), selectivity and signal shape. Based on the above assumption, the lack of fit for the matrix decomposition was improved to 7.6% which is much better than the error observed for MCR-ALS analysis without potential peak correction step.

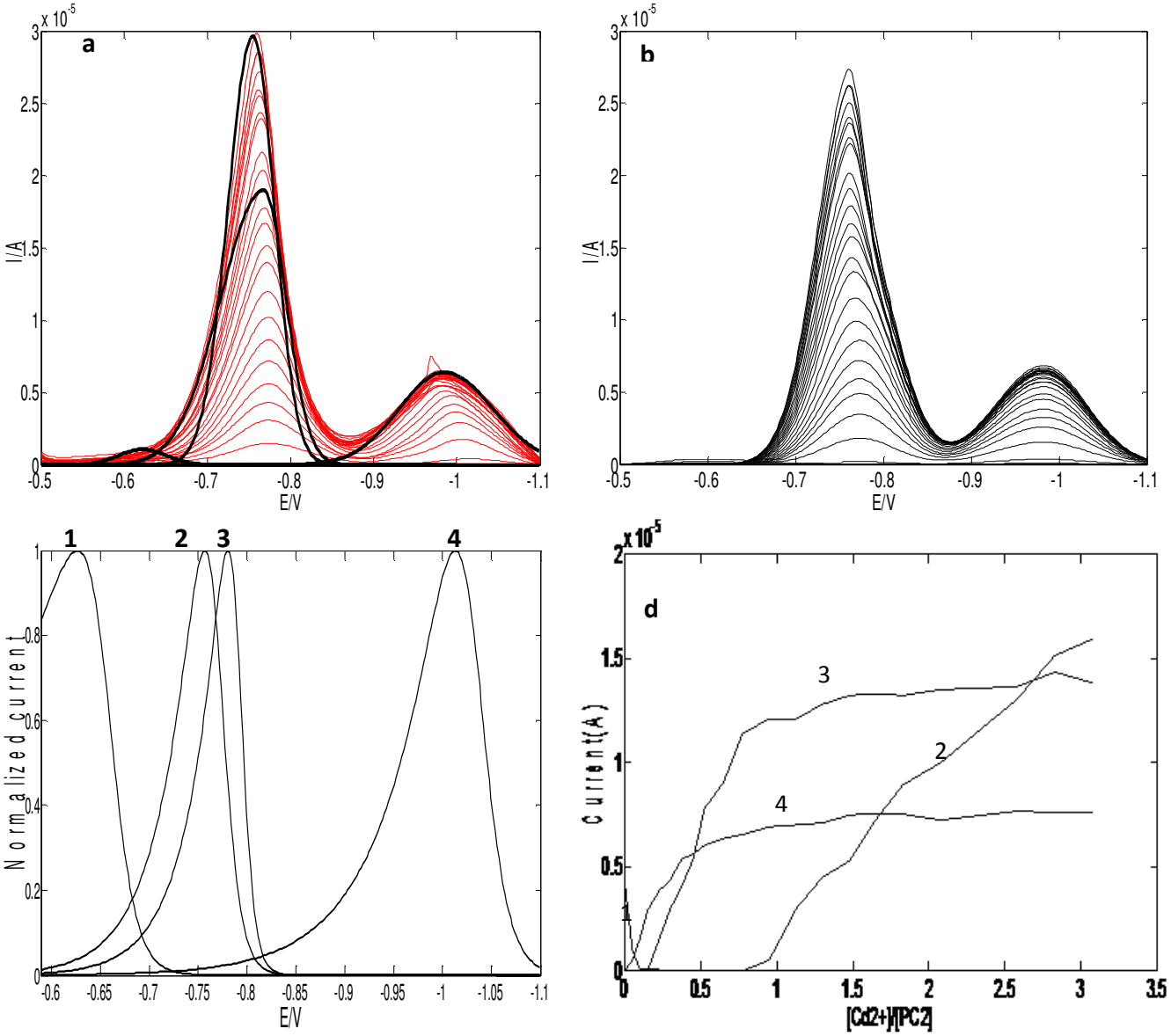

Figure 29. Analysis of the experimental data matrix produced from titration of $2x10^{-5}$ M PC_2 with $1x10^{-3}$M Cd in 0.05 borate buffer solution at pH 7.5 ; (a) estimation of the initial reference voltammogram from the experimental data matrix (b) the application of shiftfit/shiftcalc for correction of signals movements in the potential axis to produce corrected matrix (I_{corr}) (c) application of MCR-ALS for the decomposition of I_{corr} into pure voltammograms and (d) concentration matrix (C).

Figure 29 summarizes the results of MCR-ALS decomposition obtained together with the shift correction. The pure signals (Figure 29c) are named according to the position of their signals and, as stated previously from the free ligand and metal study, component 1 and 2 correspond to the anodic signal of the electrode material and free metal reduction signal, respectively, whereas component 3 and 4 correspond to the reduction of cadmium complexed with the peptides in two different ways [51]. The evolution of their concentration profiles is depicted in Figure 29d and the complexation process is explained as follows.

As stated previously it is observed that the anodic oxidation of the electrode material decreases with addition of Cd^{2+} (component 1). In the beginning of the titration where the peptide concentration is very high Cd^{2+} ion are believed to form 1:2 Cd-PC_2 complexes as observed from the fast increase of this complex concentration (component 4) until metal-ligand concentration ratio around 0.5, where it reaches a plateau (Figure 29 d). This observation confirms that the stoichiometry of the complex involves two peptide units per metal ion. The continuous movement of the peak potential of the reduction signal of this species (Figure29 a) implies the lability of the complex. Though at low cadmium-to-ligand ratios 1:4 stable compelexes where Cd^{2+} ion coordinates tetrahedrally with four sulfur atoms of different peptides were observed as a dominant species by 1H-NMR [53], XAFS [19] and some electrochemical methods [25] under the experimental condition of this study this species could not be observed. The results of ESI-MS experiment at lower metal– to-ligand ratio also proved the absence of this complex species. Starting from the ratio of 0.25 the signal related with another complex starts to be observed which stabilizes at the ratio 1.0 (component 3). Therefore the stoichiometry of this will be 1:1, where one cadmium ion binds with at least one sulfur group of the peptide. The formation of 1:1 Cd-PC_2 complex was observed starting from Cd-PC_2 ratio of about 0.25 before the 1:2 Cd-to-PC_2 formation is completed at around 0.5 Cd-to-PC_2 ratio. This suggests the simultaneous formation of 1:2 and 1:1 complexes instead of successive complex formation. At metal-to-ligand ratio higher than around 1.0, where component 3 is stabilized, the free metal reduction is observed which is indicated by the linear increment of component 2.

The matrix (Irep) which resembles the experimental matrix (Iexp) was produced from the product $\mathbf{CV^T}$ of the matrices obtained by MCR-ALS shifted by the shiftcalc program by using the optimal ΔE values. In general a good reproduction of the experimental matrix was possible

from the result of MCR-ALS analysis. The error distribution along the potential axis is shown in Figure 30(c).

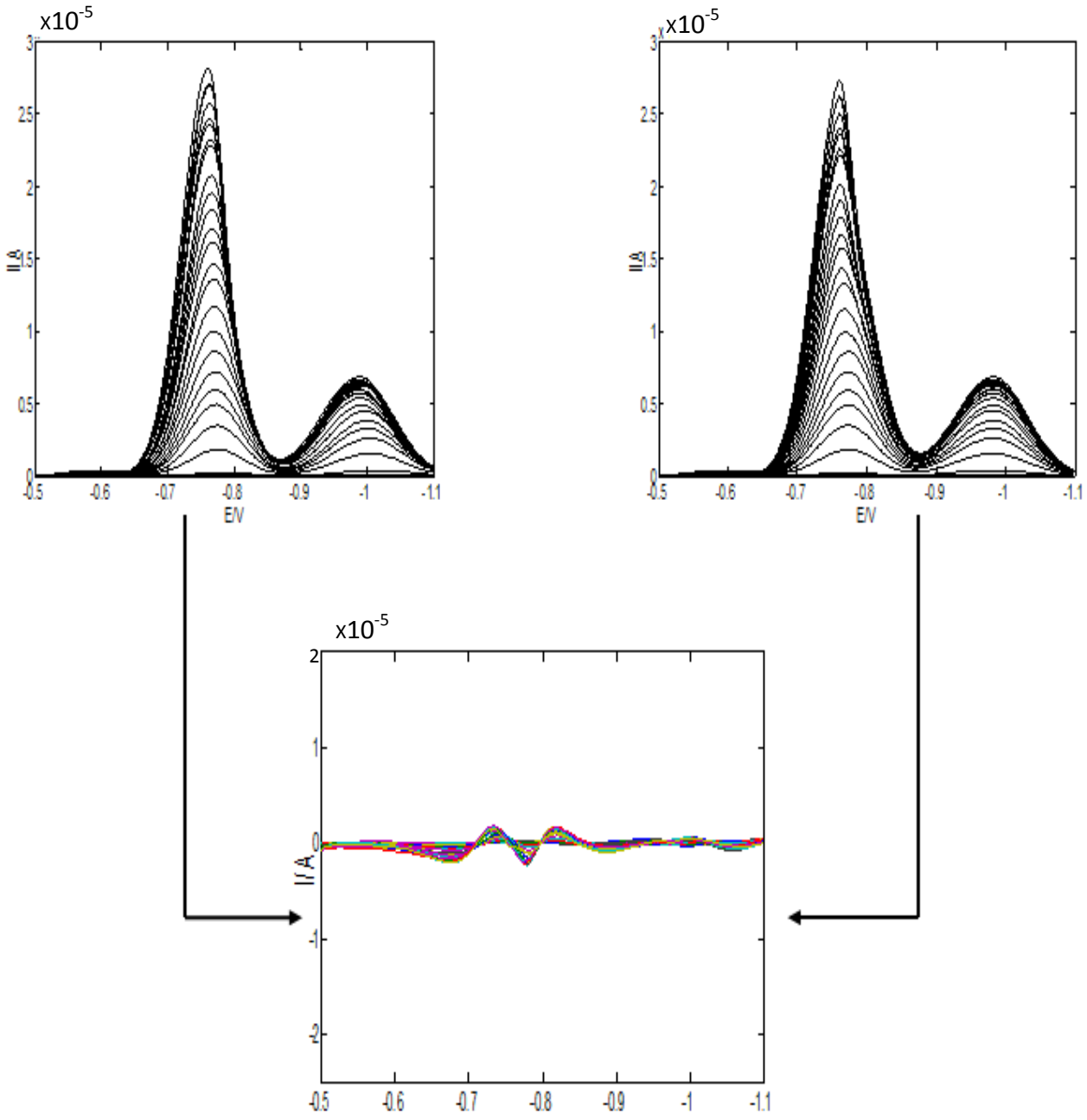

Figure30. Error distributions along the potential axis(c) as a result of the deviation of the reproduced marix , Irep (b) from the experimental matrix Iexp (a)

It can be seen that an appropriate choice of the reference signals (Fig. 7b) produces a set of potential shifts and current decreases (Fig. 7e) with a good reproduction of the original data matrix (Fig. 7d), confirmed by a reasonable lof (6.7%).

ESI-MS experiments at various metal to ligand concentration ratios were done to detect the available complex species in a particular solution with the view to get supportive information for the complexation sequence suggested from the result of voltammetric method above.

Molar ratio of the components (Cd:PC_2)	m/z + ve(int.%)	Assignment
1:4	538.33	$[PC_2+H]^+$
	1191	$[Cd(PC_2)_2-H]^+$
	1301	$[Cd_2(PC_2)_2 -H]^+$ (minor)
1:1	538.33	$[PC_2+H]^+$
	652.013	$[Cd(PC_2)-H]^+$
	1191	$Cd(PC_2)_2-H]^+$
	761.9	$Cd_3(PC_2)_2-H]^+$ (minor)
2:1	538.33	$[PC_2+H]^+$
	652.013	$[Cd(PC_2)-H]^+$
	1410	$[Cd_3(PC_2)-H]^+$ (minor)

Table 4. ESI-MS data obtained for solutions containing PC_2, Cd(II), and Bi(III) in various proportion in 1:9 acetonitrile: 20 mM ammonium acetate in water at pH 7.5.

For a solution containing 1:4 Cadmium-to-PC_2 the dominantly observed species were $Cd(PC_2)$ and $Cd(PC_2)_2$ as shown in Table 4 which support the finding for the existence of both 1:1 and 1:2 complexes at lower metal–to-ligand ratio. Quantitative comparison of the peaks appearing for those complexes in the various sample solution was difficult from the main mass spectra spectrum due to the large peak associated with the uncomplexed PC_2 that obscure the rest of the peaks. However magnification of the individual peaks in the spectrum reveals that Cd_2PC_2 and $CdPC_2$ are the dominant species for higher metal concentration while $Cd(PC_2)_2$ species was

observed to be dominant at equal metal-to-ligand ratio and at relatively higher ligand concentration. However significant amount of polynuclear metal complexes were not detected in any sample solution.

CONCLUSIONS

4. CONCLUSION

The present study demonstrates the suitability of BiSPCE for metal speciation studies of Cd^{2+} in the presence of thiol rich peptides with possibility of using MCR-ALS in some cases.

The absence of signal splitting, wider linearity, and higher sensitivity was observed for metal determination as compared to BiFE which support the suitability of BiSPCE for metal speciation studies in relatively wide concentration ranges especially for metals whose reduction signal splits on the use of BiFE that include Cd^{2+} and Zn^{2+}. The other improvement observed on the use of BiSPCE is that the anodic signal associated with the free ligand was significantly weakened in comparison to its existence on the mercury electrode. In addition the entire set of signals observed using this electrode were well separated in most cases and this may allow a qualitative and quantitative analysis of the complexation sequence without the need of chemometrical support. The other important fact observed is that BiSPCE showed a very high sensitivity to the free metal reduction signal which implies the suitability of this electrode for metal determination investigations. Although the reproducibility of this electrode was lower than the corresponding BiFE its use for such speciation studies is still reasonable.

From the results of ESI-MS experiment, extensive complex formation of Bi(III) with thiol peptides was observed, which restricts the use of in situ mode of Bi-film electrode preparation from a solution containing Bi(III), peptides and the analyte metals. Consequently in this study Bi-film was prepared on the screen printed carbon electrode in ex situ mode and gave consistent results with previous studies made on Cd^{2+} metal complexation with SH-bearing compounds on the conventional mercury electrode.

From optimization of the experimental conditions it was found that the best condition was pH 7.5 in 0.05 borate buffer solution, which gave a wide working potential window and a relatively good signal improvement for the reduction of the complexes species. Due to the inert electrochemical character of Cd-GSH species on the bismuth film electrode the complexation study at lower metal and peptide concentration ($< 10^{-5}$ M), which is the useful range for further chemometrical data analysis, was not possible. For this reason adsorptive accumulation of the complex species on to the film electrode was

applied to achieve the study of complexation process at lower peptide and ligand concentration. However no significant improvement of the signal intensity of the reduction of those complex species was found and also a well established relationship between the concentration of species and their respective signal intensity was not observed. Therefore complexation process was done at higher metal and peptide concentration for the purpose of qualitative interpretation of the complexation sequence for GSH-Cd system. In contrast, a relatively better signal intensity was obtained for complexes associated with Cd-PC_2 therefore MCR-ALS was applied to extract information about the stiochiometries of the various Cd-PC_2 complexes formed after the necessary data correction.

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